

DECLARATION APPENDIX - A

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Report

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Introduction

The present report summarizes the analysis which have lead to the detection of abnormal protein clusters (combinatorial protein patterns, CPP) on the cell surface of peripheral mononuclear cells (MNC) in ALS. These clusters are both the first biomarker of ALS and the rational for new therapeutic strategy interfering with these clusters which are all associated with the FcγRIII Receptor (CD16). These FcγRIII-associated clusters are part of the ALS Toponome¹.

The first part of this report gives an overview on the concept of the toponome and on the technology that underlies these analysis which enables for the first time protein fingerprinting on the single cell level (toponomic fingerprinting). The second part describes the methods used in this study and the third part describes the results including the visualization of the abnormal protein clusters in ALS.

Summary

In order to analyse the cell surface differentiation status of peripheral blood mononuclear cells (MNC) in ALS Toponomic Fingerprinting with a library of 18 different antibodies was performed.

Toponomic Fingerprinting is a technology that allows for the first time to trace out protein networks on the single cell level.

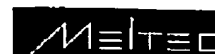
In the 2D approach the protein expression was visualized at the single cell level in the 2D mode. After setting a protein-specific threshold, protein combinations could be expressed as binary codes (1 = protein is present above threshold, 0 = protein is present below threshold). To detect disease specific protein combinations, MelTec screens for a common denominator, the so called CPP-motif (see methods). For example, the CPP-motif "01**" denotes all protein combinations where protein 1 is expressed below threshold (0) and protein is above threshold (1), while each of the other two markers can be present or absent (*). Cells can theoretically combine n screened proteins in 3ⁿ different CPP-motifs. Screening 18 cell surface marker proteins allows for 387 Mio. possible motifs. Comparing MNC's from healthy volunteers and ALS patients both groups show statistically significant differences (p < 0.025) in 140 CPP-motifs. All abnormal ALS-specific CPP-motifs contain CD16 (FcγRIII) as the leading protein and therefore form ALS-specific super-motifs.

To validate the 2D-data we performed a 3D analysis by imaging the cells in 500nm overlapping z-stacks. Per MNC 13 optical sections were obtained by deconvolution. The 3D images verify the ALS-specific CPP motifs, i.e. abnormal cell surface protein clusters, containing CD16, indicating an abnormal cell surface differentiation.

On the basis of the here presented 2D and 3D data a peripheral biomarker for ALS and basis for CD16-oriented modification therapy has been established.

¹ Toponome: "Topos" (greek: place) and Nomos (greek: law), the entirety of all proteins, protein-complexes, and protein networks traced out directly on the single cell level in the natural environment of the cells in situ. W. Schubert, Topological Proteomics, Toponomics, MELK-Technology. In: Hecker, M., Möllner, S. (eds.). Proteomics of Microorganismus: Fundamental Aspects and Application, Advances in Biochemical Engineering/Biotechnology. Springer Verlag, Berlin-Heidelberg-New York (In press)

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The concept of the Toponome

In earlier work (see chapter toponomic fingerprinting) a whole cell protein fingerprinting technology (WCPF)/MELK) was developed, that allows for the first time to analyse quantitatively protein networks and protein complexes at the single-cell level, in the natural environment of cells *in situ* (e.g. in tissue) by tracing out hundreds of proteins at the subcellular level (essentially) simultaneously. By using large tag libraries to label any proteins in cells simultaneously² the technology unravels the hierarchy of proteins and protein complexes assembled in the cell's toponome. By definition, the toponome³ is the entirety of all proteins, protein-complexes, and protein networks traced out directly on the single cell level in the natural environment of the cells *in situ* (e.g. tissues) by *whole cell protein fingerprinting*. The technology addresses the fact that each protein must be at the right time at the right place at the right concentration in a cell to interact with other proteins assembled in a spatially organized network. To encode the myriads of cellular functionalities, cells appear to having at their disposal a large, albeit finite and highly non-random repertoire of toponome units (TU's), i.e. a system of rules to construct topological hierarchies in a cell's proteome. The entirety of these TU's represents the total functional code of a cell. The complete *toponome* is therefore as fundamental a data set as the *genome* or the *proteome*.

Deciphering the human toponome is a necessary next step in functional genomics. It is now feasible by combining whole cell protein-fingerprinting (WCPF/MELK) technology, experimental cell models, and functional protein-linkage analyses, yet requiring new data-mining, statistical, and mathematical strategies involving e.g. a fusion of combinatorial geometry and stochastics.

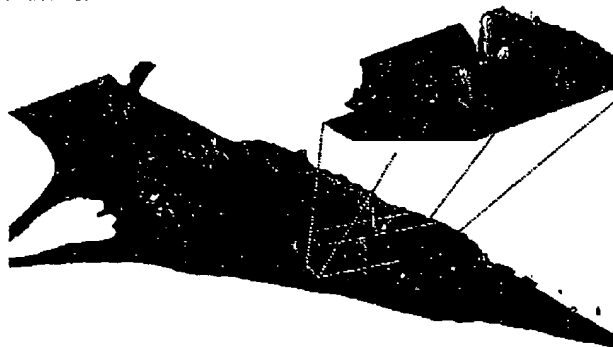


Fig. 1 gives an example of a toponomic fingerprint of a cell displaying a network of multiple subcellular protein complexes assembled within defined subcellular sites (ER, Golgi, early and late golgi vesicles, a.s.o.). Note that every possible functional state is characterized by specific topological arrangement of subcellular protein complexes that can be observed directly by *whole cell protein fingerprinting* in thousands of cells simultaneously (Toponome Mapping Factory).

Given the cell, we can distinguish at least four distinct functional levels:

genome, transcriptome, proteome, and toponome. The information content increases dramatically at each level (Fig. 2). In order to properly address cell functionalities, it is necessary to analyse the toponome, because (i) it integrates proteins and their various modifications into 3D networks, (ii) allows to obtain information on rapid functional changes due to translocation and topological rearrangement of proteins (i.e. rapid biophysical

² I.e. Schubert, W.: US-Patent 6,150,173, "Automated Determining & Measuring Device & Method"

³ Toponome: "Topos" (greek: place) and Nomos (greek: law). W. Schubert, Topological Proteomics, Toponomics, MELK-Technology. In: Hecker, M., Müller, S. (eds.), Proteomics of Microorganisms: Fundamental Aspects and Application, Advances in Biochemical Engineering/Biotechnology. Springer Verlag, Berlin-Heidelberg-New York (In press)

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processes like diffusion, collision, protein assembly, disassembly, a.s.o.) and (iii) represents (the result of applying) all the *rules* that cells use to form the highly non-random 3D organisation of the proteome that enciphers the myriads of cell functions.

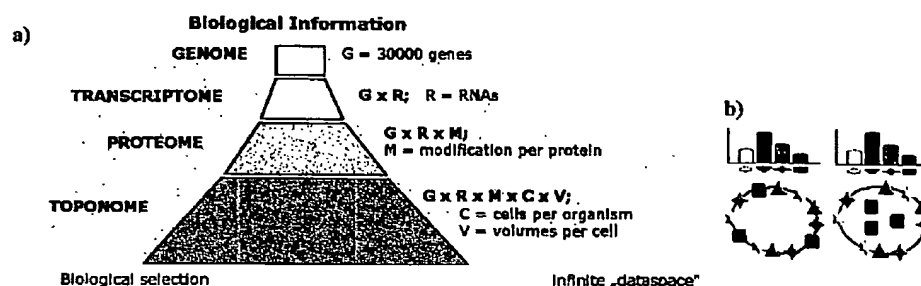


Fig. 2 a) Schematic illustration of the different functional levels of the cell and the related increase of information content; b) Note that different functional codes of the cell represented by different subcellular protein networks (2 cells, bottom images), cannot be recognized by expression profiling proteomic techniques, because the abundance of the single proteins is identical in both cells and averaging over extracted proteins allows no conclusion on the single cell level (upper 2 images)

Given the peripheral mononuclear cell (MNC), the MELK approach allows for the first time to analyse the toponome of cell surface proteins. Thereby it is possible to identify abnormal combinations of proteins at the cell surface, that would otherwise not be conspicuous because their abundance and/or regulation, captured by conventional proteomics or transcriptomics technologies is not abnormal. Therefore the MELK technology kept as the unique technology to quantify the toponome of the cell surface.

Toponomic Fingerprinting: Technology Overview

Principle of Toponomic Fingerprinting (TF)

The technological principle of TF is based upon the theory that a cell is made up almost entirely by protein-complexes in all compartments and also in the cytosol. Earlier data suggested⁴ that this allows proteins to diffuse freely between these complexes with a kinetic almost like in water although the average concentration of proteins in a given compartment may be semicrystalline. By this highly conserved structural principle the internal environment of the cells provide a stable diffusion kinetic for any protein, for example for newly synthesised proteins across defined water channels. Provided that (i) this all over protein structure of a cell can be preserved by specific "fixation" methods, (ii) externally applied tag molecules, like antibodies, can penetrate the cellular membranes, and (iii) the binding of a tag molecule to a specific target molecule within the cell can be detected optically, it is possible to localize any number of protein species (or other molecular classes) in a cell by using large tag libraries applied by an imaging robot (Appendix-Fig. 1). Since the tag molecules can diffuse freely through the cellular "water channels" and recognise their target molecules in the cell, we call this diffusion principle "Successive Ligand Aggregation Stable Diffusion Channelling (SLASDIC)". Using SLASDIC we address the toponome which is the entirety of proteins, protein complexes and protein networks traced out directly on the single cell level. It

⁴ Schubert, W. EJC, 58, 395-410 (1992)

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is held that the toponome enciphers the complete functional plan of a cell or a tissue. It is composed of ~~highly non-random spatial assemblies of proteins~~, the functional toponome unit (FTU's). There are clear-cut rules for the local formation of FTU's in cells, which, when deciphered by TF at a large scale, unravel the hierarchies of proteins and directly lead to the detection of key target proteins. This functional organization of proteins is inaccessible when the cellular structures are destroyed, as for example by large scale expression profiling procedures.

All steps from the robotic generation of primary toponome data to the detection of functional toponome units follow highly standardized protocols and have been fully automated (Appendix-Fig. 2).

Data acquisition

Briefly, by a two-step water extraction of cells the proteins, protein complexes and protein networks in the cell are highly stabilized in situ. By this same procedure the cellular membranes are made penetrable for tag libraries, like antibody libraries. In most cases monoclonal antibody libraries are used to tag proteins within the cell. Each antibody of the library is conjugated to one specific dye, for example FITC. This dye-conjugated library is then calibrated according to antibody concentration, tissue or cell type of interest, and optical parameters of the robotic set up. All the following steps are fully automated and performed by the imaging cyler MELK (Multi-Epitope-Ligand-"Kartograph"). One single MELK robot (Appendix-Fig. 1) consists of a multi-pipette handling unit, a modified upright or inverse epifluorescence microscope, and a sensitive CCD imaging system. Multiple MELK robots can be assembled to cooperative networks for "massive parallel toponome screening". The device permits the automated running of Repetitive-Incubation-Imaging-Bleaching Cycles (RIIBC) on any type of biological sample, a single cell or thousands of cells simultaneously, or tissue sections. Briefly, a first dye-conjugated AB is incubated (Appendix-Fig. 1); after several washing steps the fluorescence image is registered and stored; the fluorescence signal is then destroyed by "soft" diffuse multi-wavelength excitation avoiding any energy transfer-induced alteration of the proteins in the cells; after registration of the resulting "endpoint" image (post-bleaching image) the second cycle (RIIBC) is automatically started by applying the second dye-conjugated antibody, and so forth. RIIBC can involve a large number of antibodies, clearly hundreds, to tag proteins in cells. The AB's applied to the sample remain bound in situ and, according to SLASDIC, do not hinder the binding kinetics of other antibodies applied thereafter. This can be proven by means of repetitive runs, when the antibodies are used at an undersaturated concentration. Appendix-Fig. 3 gives an example of repetitive runs using a library containing 18 different AB's against cell surface receptor proteins of lymphocytes. After run 1 the same AB library at the same AB concentration is applied a second (run 2) and a third time (run 3) to the same sample. As illustrated by direct comparison of the corresponding epitope-signals in the different runs (Appendix-Fig. 3, horizontal collection of images), the subcellular location of corresponding protein signals in run 2 and run 3 are identical, although the intensity of the signals decline from run 1 to run 3, as expected, according to the progressive antibody saturation of remaining free epitope binding sites. In order to illustrate the fact that a progressive "load" of the cell surface by antibodies applied in run 1 does not hinder the subcellular specificity of binding of AB applied in run 2, one cell denoted as cell 1 in Appendix-Fig. 3 is depicted. The cell surface of this cell shows 4 characteristic toponome units (TU) by differential abundances of different cell surface receptor molecules thereby uncover 4 different functional plasma membrane domains. Appendix-Fig. 4 shows the pixel plot of the antibody No. 1 of the depicted cell 1 in Appendix-Fig. 3. This antibody labels cell 1 in defined pixels in run 1 (Appendix-Fig. 4 red bars). The green and the blue bars illustrate the decline of the fluorescence intensity in run 2 and run 3, respectively, whilst the topological patterns of labelled pixels of the three runs are

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identical. This indicates, quantitatively, that the AB No. 1 repetitively recognises the same subcellular sites substantiating the SLASDIC principle. It has been demonstrated by numerous internal protocols that SLASDIC is given in all human tissues and cell types examined so far, i.e. approx. 1000 human samples in the years 1990-1997.

Image Processing

The steps of image processing involve proprietary robust software tools for image correction, threshold setting, signal alignments, and object segmentation, finally leading to clearly defined objects, which can be segmented towards non-objects (i.e. background). Because of limited space these algorithms are not further detailed here. Briefly, alignment of signals is shown in Appendix-Fig. 5, providing the possibility to map cellular or subcellular toponomic fingerprints.

Data mining and nomenclature

a) Cellular and subcellular TF

Cellular TC (cTF) are schematically illustrated in Appendix-Fig. 5, c. They are defined as a characteristic collection of cellular combinatorial protein patterns (cCPP) composed of single fluorescence intensities, which correspond to single labelled proteins quantified as an integral over the cell. Typically, TF are highly specific for a cell type, a functional state of a cell (such as in disease) or a tissue. As illustrated in Appendix-Fig. 5c, these cCPP are highly heterogeneous, but this heterogeneity is non-random. By definition subcellular TF (sTF) are a characteristic collection of subcellular CPP (sCPP) containing protein clusters with improved spatial resolution. Subcellular TF are illustrated below for hepatocytes in culture (see biological example I).

b) Visualisation and quantification of toponomic fingerprints

Appendix-Fig. 6 illustrates schematically a simple visualisation method for TF comparing two different cell systems. Visualization of TF in two-dimensional plots (fingerprints) is a straight forward solution. All CPP's within the TF are expressed as a combinatorial binary code in which each protein is denoted as absent or present above a threshold level (0/1). A fixed "reading frame" (x-axis) contains all theoretically possible CPP's. The CPP as a binary code can be transformed into a decimal number on the x-axis. This decimal number is plotted against the frequency of the CPP's (y-axis) in the sample. The resulting TF contain all occurring CPP's as well as "silent areas", which indicate that the corresponding theoretical CPP's on the reading frame do not occur in the biological system measured. The combination of occurring CPP's and silent areas together make up the TF of a cell system. These TF pinpoint differences between treated and untreated biological samples, or between different cell systems or diseases. Note the differences of TF between cell system 1 and cell system 2 (Appendix-Fig. 6). Matching these TF reveals a difference map containing the CPP-Clusters, which are specific for the given cell system. By detailed analysis of these system-specific CPP-clusters one protein common to all CPP's within the cluster, is identified. This protein is called the "leading protein" (protein 1 in Appendix-Fig. 6). In our example this protein is inversely correlated with protein 2 (always absent) and variably associated with proteins 3 to n (wild cards). We call this constellation a CPP-motif. This CPP-motif represents the hierarchy of proteins, among which the leading protein is the key player (potential target protein). We have developed an algorithm called "motif-finder", which allows us to automatically identify CPP motifs with high statistical significance among different comparison groups, for example, control group and treatment groups. Assuming that MELK-

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measurements were made of M treated and N untreated biological samples, the Data-Mining screens all possible motifs for significant differences. This is done by comparing the relative frequencies of a motif in the treatment group with the relative frequencies of the same motif in the control group using the standard student's t-test or the more robust Wilcoxon Sum Rank test. The output is a set of CPP-motifs characterizing the difference between the treatment and the control groups.

We have also developed in collaboration with external groups two further independent methods to detect highly significant functional linkages of topological associations of proteins. One approach we call "Subset Surprisology" (research group around A. Dress, Univ. of Bielefeld). The other algorithm is based upon a specific artificial neuronal architecture (research group around T. Nattkemper and H. Ritter, Univ. of Bielefeld).

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Biological example I

Two-dimensional toponomic fingerprinting of Hepatocytes in culture

This example illustrates a 2D-toponomic fingerprinting approach to hepatocytes in culture with subcellular resolution. Control cells and treated cells are compared. We have mapped simultaneously 23 different proteins present in different compartments of the cells. Out of a large data set we illustrate here the distribution of the 30 most frequent sCPP's coded in different colours. Appendix-Fig. 7 gives the list of the most frequent sCPP's out of 105.732 occurring CPP's in the control group and 82.560 CPP's occurring in the treated cells. Note that the simultaneous mapping of 23 proteins at binary coding (0/1) comprises a theoretical possible maximum number of 8.388.608 CPP's. Hence the present toponome data set reveals 1.26 % and 0.98 % of the maximum number of CPP's in the control cells and the treated cells, respectively. Appendix-Fig. 8 shows the comparison of control and treated cells demonstrating substantial quantitative and topological differences between these two groups. By "massive parallel toponomic fingerprinting" the differences can be precisely quantified by TF-line plots (not shown here). Appendix-Fig. 9 gives a detail of the control cells. Appendix-Fig. 10 gives two details of the treated cells. Appendix-Fig. 9 illustrates precise subcellular landmarks of, for example, the outer surface and the more internal parts of the plasma membrane of a control cell outlined by protein P 19 (glycocalix) and P1 + P19 (unit membrane). By contrast Appendix-Fig. 10 (upper image) representing treated cells, illustrates the molecular architecture of the plasma membrane of a cell extension (treated cell), which is different to the untreated cells by showing solely protein P1, but absence of protein P19. This indicates selective absence of specific glycocalix-substructures at the latter sites. Appendix-Fig. 10, lower image, illustrates simultaneously the clustering of proteins P1 + P19, and P1 + P10 present at the tip of growth cones formed by cells under the influence of treatment. This latter event is due to abnormal translocation of unrelated proteins (specific treatment response).

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Biological example II

Three-dimensional mapping of toponomic subcellular "landmarks" of hepatocytes in culture as a basis for subcellular mapping of functional protein complexes and their annotation to compartments

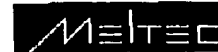
The Appendix-Fig. 11 through Appendix-Fig. 16 give defined steps of the generation of 3D images of six simultaneous subcellular "landmark" molecules in hepatocytes. These landmarks, which can principally be extended to dozens of more landmark molecules, are the basis for "reading in" hepatocyte-specific protein complexes in defined subcellular sites. These landmarks form the basis for the deciphering of the hepatocellular toponome. Technically, the present example shows the ability to map intracellular as well as surface marker molecules simultaneously: Appendix-Fig. 11 gives the overview; Appendix-Fig. 12 shows the optical section through the intracellular distribution patterns of 6 subcellular markers. Appendix-Fig. 13 shows one single optical section illustrating a deconvolved image of each single marker molecule-pattern; Appendix-Fig. 14 gives the 3D composition of the whole stack (Appendix-Fig. 12). The Appendix-Fig. 15 and 16 give two sectional views of the 3D image.

Biological example III

Three-dimensional toponomic fingerprinting to detect protein-complexes in tissue sections of the spinal cord (results of a feasibility study to map protein complexes in defined subcellular sites)

The Appendix-Fig. 17 and 18 substantiate the principle ability of TF to detect protein complexes in situ. The example shows the simultaneous mapping of thousands of single synapses in the spinal cord. Appendix-Fig. 18 shows a detail of Appendix-Fig. 17 and directly denotes presynaptic and postsynaptic protein-patterns. The postsynaptic protein complexes are formed by NMDA and NMDA-related protein families. Note that this approach can involve at least hundred proteins simultaneously enabling the analysis of the local rules specifying synaptic "qualities". This approach is a powerful tool in conjugation with biological experiments. This successful feasibility study provides strong evidence for the ability of TF to map protein networks on the single cell level in situ. We expect that these experiences to be applicable to thorough mapping the hepatocellular toponome applying selected tag libraries.

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Methods

Peripheral blood nuclear cells (MNC) were collected from venous blood of 7 healthy individuals of 7 healthy individuals and from 9 sporadic ALS patients using Ficoll-gradient isolation procedures. Cells were then diluted to a concentration of 3.5×10^6 cells in PBS, placed on cover slips and subjected to a two step water extraction procedure in order to stabilize the cellular proteins.

The cells were measured in the MELK robots using proprietary protocols which guarantee high reproducibility of the results.

Image Processing 2D

Raw images of every run underwent evaluation by a scientist to ensure that the run meets MelTec's quality standards before entering into image processing. Image processing starts with image correction. It mainly consists of background correction and normalisation of the dynamic range of intensities.

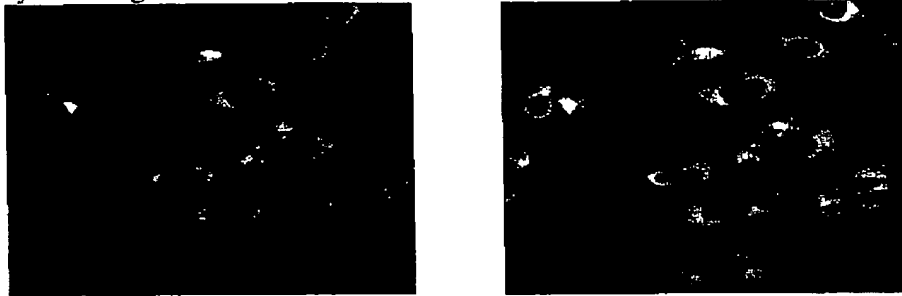


Fig. 3 Fluorescence image before and after corrections. Illustration of control cells

A pre-requisite to generating toponome fingerprints is designating a binary code to each marker for each pixel where:

- 1 indicates that the marker is present above the threshold level
- 0 indicates that the marker is not present above the threshold level

The threshold is set by MelTec's threshold algorithm, which is based on robust statistical properties of the images. The method ensures threshold above the noise floor of the image. It is possible to choose different threshold levels.



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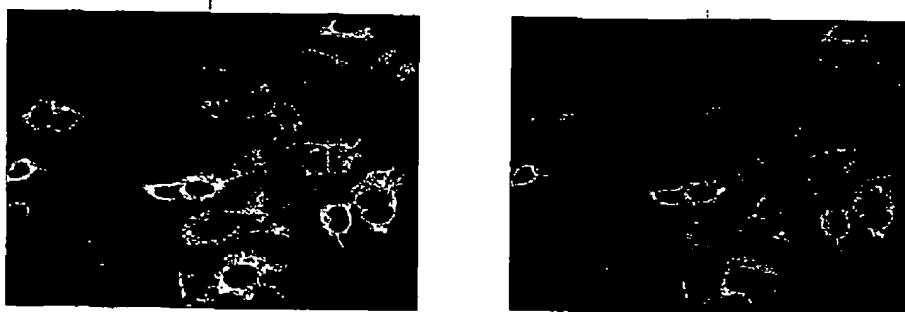


Fig. 4 Different threshold levels: Fluorescence image (top left), Binary images: low threshold (top right), medium threshold (bottom left), high threshold (bottom right).

In a last step of image processing, areas of interest are defined using markers, which are specific for different compartments (e.g. nuclei, mitochondria, cytoplasm etc.), and advanced image segmentation algorithms.



Fig. 5 Segmented image (white: background, yellow: cytoplasm, black: nuclei).

Image Processing 3D

For 3D-imaging of ALS and control MNC z-stacks of 8 consecutive sections (500nm layer) for each marker have been obtained. The 500nm steps were overlapping. The images have been processed as described under "Image Processing 2D". Additionally all images have been corrected for the bleaching effect and underwent deconvolution.

From Images to CPPs and CPP-Motifs

With a binary code, cells can theoretically combine the n screened markers in 2^n different combinatorial protein patterns (CPP). An example of a binary code (CPP) is shown in the following table:

	Marker 1	Marker 2	Marker 3	...	Marker N
	Yes	No	Yes		Yes
	1	0	1		1

Table 1 From threshold to binary codes.

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All pixels of a run or a set of runs are analysed concerning their specific CPP (s-CPP). Then the s-CPPs can be summarized in a code table, by listing the specific binary codes (s-CPPs) and their absolute or relative frequency.

Code-Table of one Run:

s-CPP	1	1	1	1	0	0	1	1	0	1	4031
s-CPP	1	0	1	1	0	0	1	1	0	1	2738
...											...
s-CPP	1	1	0	1	0	0	1	0	0	1	40

Table 2 Code table of one run.

In order to find relevant protein combinations, MelTec screens the s-CPPs for a common denominator, a so called CPP-motif. For example, the motif '01**' denotes all combinations where marker 1 is absent (0) and marker 2 is present (1), while each of the other two markers can be present or absent (*). As for CPPs, CPP-motifs can be counted and used for statistical analysis comparing a control with a treatment group. The steps from CCPs to CPP-motifs have been summarized in Figure 6.

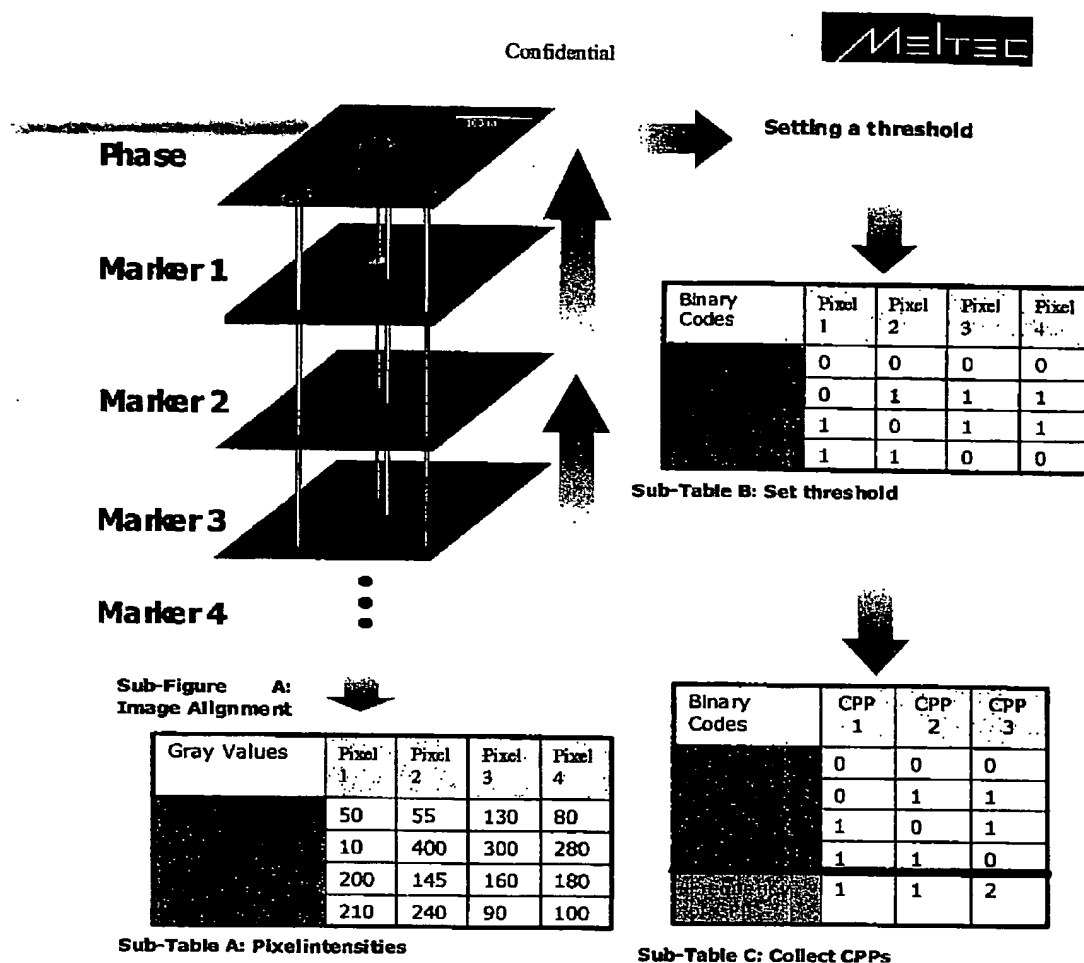


Fig. 6 Steps from image to CPP

Motif-Finder

To statistically analyse the effect of a disease on the cell surface protein toponome, the relative frequencies of CPP-motifs in control and disease groups are compared using the standard student's t-test or the more robust Wilcoxon Rank Sum test. The output of the data-mining phase is a set of CPP-motifs that characterizes the difference between the treatment and the control group.

Quality of Motifs

Two groups of motifs can be distinguished:

1. Motifs that separate the groups, e.g. control and treatment, in a paired test (lower quality)
2. Motifs that separate the groups in paired as well as in an unpaired test (higher quality).

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Motifs, which are strongly affected or newly generated in a disease, will show a statistically significant change in frequency in the paired and the unpaired test. For motifs, which are more subtly altered, differences between disease and control group can only be detected using a paired testing.

Visualisation of Results

Box plots and response plots are used to visualize results and are described below.

Box plots

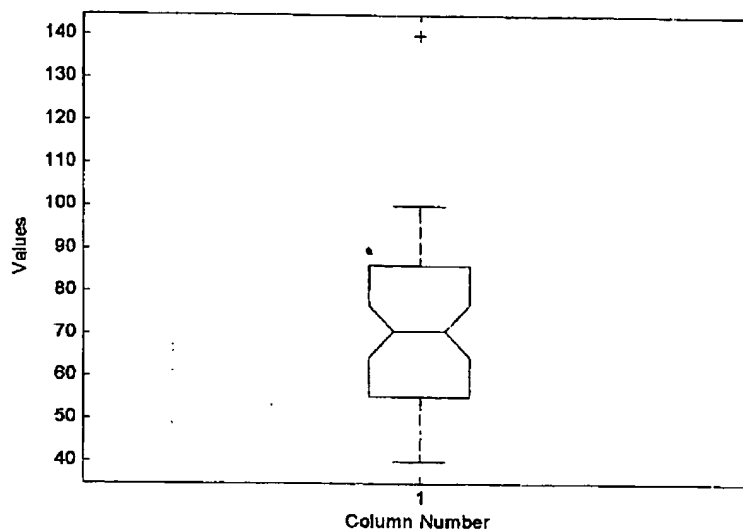


Fig. 7 Box plot.

The graph shows an example of a notched box plot. It is composed of several graphic elements:

- 1) The lower and upper lines of the "box" are the 25th and 75th percentiles of the sample. The distance between the top and bottom of the box is the interquartile range.
- 2) The line in the middle of the box is the sample median. If the median is not centered in the box, that is an indication of skewness.
- 3) The "whiskers" are lines extending above and below the box. They show the extent of the rest of the sample (unless there are outliers). Assuming no outliers, the maximum of the sample is the top of the upper whisker. The minimum of the sample is the bottom of the lower whisker. The plus sign at the top of the plot indicates data points, which are more than 1.5 times the interquartile range away from the top or bottom of the box.
- 4) The notches in the box are a graphic confidence interval about the median of a sample.

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Results: abnormal cell surface protein cluster in ALS

Identification of abnormal protein clusters on the cell surface of peripheral mononuclear cells in ALS

In order to analyse the cell surface differentiation status of peripheral blood mononuclear cells, we have used the MELK robotic approach (US-Patent, 6.150.173, 2000). This study was motivated by preliminary data suggesting that abnormal cellular phenotypes of the immune system might be present in the blood of ALS.

Application of the MELK technology included the use of a library of 18 different monoclonal antibodies directly conjugated to a dye (FITC) as outlined in the method section. These antibodies were all directed against well characterized cell surface proteins, most of them belonging to cell surface adhesion receptors and cell surface proteolytic enzymes. Table 3 gives the summary of these molecules and the specification of the monoclonal antibody library.

Tab. 3: Specification of proteins

protein (p), lectin (l)	Specification
CD2 (p)	SRBC receptor, ligand for LFA-3
CD3 (p)	CD3 complex associated with T cell antigen receptor TCR
CD4 (p)	co-recognition receptor for MHC class II with TCR
CD7 (p)	Fc receptor for IgM "FcμR"
CD8 (p)	co-recognition receptor for MHC class I with TCR
CD11b (p)	αM integrin chain of MAC-1 complex
CD16 (p)	Fcγ RIII receptor for selective binding of IgG1 and IgG3
CD19	B-cell receptor
CD26	Dipeptidylpeptidase IV, Collagen receptor
CD36 (p)	GP IV, collagen receptor
CD38 (p)	gp 45 receptor involved in leucocyte activation
CD45RA (p)	restricted leucocyte common antigen isoform containing at least exon A
CD56 (p)	neural cell adhesion molecule (NCAM)
CD57 (p)	HNK-1 receptor
CD62L (p)	L-selectin
CD71 (p)	transferrin receptor
HLA-DR (p)	MHC class II receptor
HLA-DQ (p)	MHC class II receptor

HLA = MHC

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We studied blood samples from 9 patients with the sporadic form of ALS and from 7 healthy individuals.

To analyse the cell surface Toponome data of 18 different cell surface proteins we have used a 2D and a 3D approach. In the 2D approach the cells were visualized in the 2D mode of the MELK-robotic set up. These cells were then quantified using proprietary pattern recognition-algorithms including our motif-finder (see method section). The CPP motif-finder algorithm is a powerful data mining tool to find abnormal protein clusters in diseases.

Principally, on the level of 18 different cell surface proteins, a maximum number of possible protein combinations assembled as motifs of 3^{18} (387 Mio.) can be expected, when each protein is captured as a binary value above a threshold [1/0] and wild card (*). In our data set we found 140 really occurring and highly specific CPP motifs out of 387 Mio. possible motifs ($p < 0.025$).

Table 4 gives the total list of ALS-specific motifs, i.e. abnormal cell surface protein clusters in ALS related to most significant abnormal cell surface protein clusters in ALS.

Table 4

Decimal code	Proteins	Motif	Base Motif ¹	P-Value	MV-ALS	MV-Contr	MVT - MVC	Ratio (MVT MVC)
3 15	hla-dr+ cd11b-	***G*****G*****	*****G*****G*****	0.012452262	0.0479	0.0312	0.0168	1.6321
5 13	cd3- cd62+	***G*****G*****	*****G*****G*****	0.016641101	0.0453	0.0284	0.0170	1.5993
13 18	cd62+ cd2-	***G*****G*****	*****G*****G*****	0.020480801	0.0416	0.0260	0.0155	1.5882
15 16	cd11b- cd38+	***G*****G*****	*****G*****G*****	0.010659047	0.0387	0.0144	0.0243	2.6854
19 15	cd4+ cd16+ cd11b-	***G*****G*****	*****G*****G*****	0.002827836	0.0017	0.0004	0.0014	4.7657
1 12 13	cd4- cd7- cd62+	***G*****G*****	*****G*****G*****	0.018193537	0.0208	0.0100	0.0106	2.0809
2 3 9	cd8+ hla-dr+ cd16+	***G*****G*****	*****G*****G*****	0.020178040	0.0003	0.0001	0.0002	3.1030
3 4 8	hla-dr+ hla-dq- cd45ra+	***G*****G*****	*****G*****G*****	0.023331877	0.0097	0.0043	0.0054	2.2499
3 4 13	hla-dr+ hla-dq- cd62+	***G*****G*****	*****G*****G*****	0.020289597	0.0170	0.0077	0.0093	2.2178
3 7 13	hla-dr+ cd38- cd62+	***G*****G*****	*****G*****G*****	0.024286228	0.0139	0.0067	0.0052	1.6035
3 9 12	hla-dr+ cd18+ cd7+	***G*****G*****	*****G*****G*****	0.021000921	0.0010	0.0003	0.0007	3.2748
3 12 13	hla-dr+ cd7- cd62+	***G*****G*****	*****G*****G*****	0.017349630	0.0184	0.0098	0.0068	1.9170
3 13 14	hla-dr+ cd62+ cd71-	***G*****G*****	*****G*****G*****	0.016570815	0.0299	0.0176	0.0124	1.7058
4 9 15	hla-dq+ cd16+ cd11b-	***G*****G*****	*****G*****G*****	0.022759490	0.0009	0.0003	0.0007	3.2851
4 10 11	hla-dq- cd57+ cd56-	***G*****G*****	*****G*****G*****	0.024400031	0.0087	0.0045	0.0052	2.1371
4 10 15	hla-dq- cd57+ cd11b-	***G*****G*****	*****G*****G*****	0.019083918	0.0107	0.0048	0.0058	2.1812
5 13 18	cd3- cd62+ cd2-	***G*****G*****	*****G*****G*****	0.003250799	0.0325	0.0169	0.0156	1.9200
8 9 18	cd28- cd18+ cd38+	***G*****G*****	*****G*****G*****	0.018587089	0.0011	0.0002	0.0008	4.9144
8 10 13	cd28- cd57+ cd62+	***G*****G*****	*****G*****G*****	0.018385278	0.0024	0.0009	0.0015	2.7899
8 12 13	cd28- cd7- cd62+	***G*****G*****	*****G*****G*****	0.020904358	0.0246	0.0132	0.0116	1.8711
6 13 18	cd28- cd62+ cd38+	***G*****G*****	*****G*****G*****	0.014560311	0.0127	0.0054	0.0073	2.3584
7 18 18	cd38+ cd36+ cd2-	***G*****G*****	*****G*****G*****	0.009741418	0.0253	0.0085	0.0158	2.6596
9 13 16	cd18+ cd62- cd36+	***G*****G*****	*****G*****G*****	0.017258974	0.0008	0.0001	0.0008	5.4058
12 13 16	cd7- cd62+ cd38+	***G*****G*****	*****G*****G*****	0.018394408	0.0121	0.0041	0.0080	2.9473
13 14 16	cd62+ cd71- cd38+	***G*****G*****	*****G*****G*****	0.012088278	0.0259	0.0138	0.0124	1.9100
13 18 18	cd62+ cd36+ cd2-	***G*****G*****	*****G*****G*****	0.001335069	0.0185	0.0060	0.0105	2.7582
1 9 12 13	cd4- cd18- cd7- cd62+	***G*****G*****	*****G*****G*****	0.017087165	0.0203	0.0097	0.0108	2.0922
3 4 9 13	hla-dr+ hla-dq- cd18- cd62+	***G*****G*****	*****G*****G*****	0.021592519	0.0168	0.0078	0.0082	2.1984
3 9 12 13	hla-dr+ cd16- cd7- cd62+	***G*****G*****	*****G*****G*****	0.017821973	0.0183	0.0086	0.0087	1.9063
3 9 13 14	hla-dr+ cd18- cd62+ cd71-	***G*****G*****	*****G*****G*****	0.017523442	0.0298	0.0176	0.0121	1.6949
5 9 13 18	cd3- cd16- cd62+ cd2-	***G*****G*****	*****G*****G*****	0.002937682	0.0317	0.0185	0.0153	1.9278

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6 9 12 13	cd26- cd16- cd7- cd82i+	0.018668804	0.0242	0.0129	0.0114	1.8836
6 9 13 16	cd26- cd16- cd82i+ cd36+	0.014839165	0.0123	0.0053	0.0071	2.3330
7 9 18 18	cd36+ cd16- cd36+ cd2-	0.009188089	0.0250	0.0095	0.0158	2.6425
9 12 13 16	cd16- cd7- cd82i+ cd36+	0.017636128	0.0120	0.0041	0.0079	2.9410
9 13 14 16	cd16- cd82i+ cd71- cd36+	0.013138948	0.0268	0.0135	0.0121	1.8922
9 13 16 18	cd16- cd82i+ cd36+ cd2-	0.001155720	0.0163	0.0059	0.0104	2.7756
1 2 9 18	cd4- cd8- cd16+ cd2-	0.024458071	0.2475	0.5740	-0.3265	2.3193
1 9 11 18	cd4+ cd16+ cd56- cd2-	0.024454999	0.2969	0.0747	0.2242	3.9899
2 4 8 15	cd8+ hla-dq- cd16+ cd11b-	0.016171248	0.1248	0.0145	0.1103	8.8089
2 9 10 15	cd8- cd16+ cd57+ cd11b-	0.021878712	0.0808	0.0258	0.0848	3.5161
4 5 9 15	hla-dq- cd3+ cd16+ cd11b-	0.011103845	0.1588	0.0423	0.1165	3.7545
4 9 10 15	hla-dq- cd16+ cd57+ cd11b-	0.020321330	0.0945	0.0194	0.0751	4.8761
4 9 15 16	hla-dq- cd16+ cd11b- cd36+	0.018569651	0.0874	0.0139	0.0735	8.2748
5 8 9 15	cd3+ cd26- cd16+ cd11b-	0.016358878	0.1289	0.0376	0.0914	3.4351
5 9 13 15	cd3+ cd16+ cd82i- cd11b-	0.024449522	0.1059	0.0223	0.0835	4.7411
5 9 15 16	cd3- cd16+ cd11b- cd36+	0.009783943	0.1369	0.0227	0.1142	6.0325
6 8 9 15	cd26- cd45ra+ cd16+ cd11b-	0.002636177	0.5581	0.1788	0.3793	3.1455
6 9 10 15	cd26- cd16+ cd57+ cd11b-	0.020100177	0.1019	0.0201	0.0818	5.0767
6 9 12 15	cd26- cd16+ cd7+ cd11b-	0.002563635	0.4320	0.1213	0.3107	3.5818
6 9 14 15	cd26- cd16+ cd71+ cd11b-	0.020404585	0.2243	0.0222	0.2021	10.1102
6 9 15 16	cd26- cd16+ cd11b- cd36+	0.014359531	0.1483	0.0121	0.1362	12.2885
6 9 16 18	cd26- cd16+ cd11b- cd2+	0.013424306	0.2087	0.0454	0.1614	4.5589
9 12 13 15	cd16+ cd7+ cd82i- cd11b-	0.014487324	0.3452	0.1375	0.2077	2.5109
9 12 15 16	cd16+ cd7- cd11b- cd36+	0.020848893	0.0861	0.0012	0.0848	69.4071
9 13 15 18	cd16+ cd52i- cd11b+ cd2+	0.022828827	0.0039	0.0801	-0.0762	20.5718
9 14 15 16	cd16+ cd71- cd11b- cd36+	0.009118507	0.1270	0.0132	0.1139	9.6454
9 15 16 18	cd16+ cd11b- cd36+ cd2-	0.023778212	0.1311	0.0102	0.1209	12.8610
3 7 8 16	hla-dr- cd36+ cd45ra- cd36+	0.010738688	0.0074	0.0025	0.0049	2.9709
3 7 12 16	hla-dr- cd36+ cd7- cd36+	0.012943020	0.0056	0.0015	0.0040	3.6049
3 8 13 16	hla-dr- cd45ra- cd82i+ cd36+	0.011843894	0.0073	0.0032	0.0041	2.2658
3 9 13 16	hla-dr- cd16+ cd82i- cd36+	0.018558566	0.0003	0.0001	0.0002	4.0206
3 12 13 16	hla-dr- cd7- cd82i+ cd36+	0.008909445	0.0044	0.0012	0.0032	3.6049
1 3 8 9 15	cd4- hla-dr- cd45ra+ cd16+ cd11b-	0.021137247	0.4384	0.1873	0.2482	2.3306
2 3 4 9 15	cd8+ hla-dr- hla-dq- cd16+ cd11b-	0.023358682	0.1779	0.0205	0.1574	8.8603
3 4 8 9 15	hla-dr- hla-dq- cd45ra+ cd16+ cd11b-	0.004077809	0.5168	0.1461	0.3707	3.5368
3 4 9 12 15	hla-dr- hla-dq- cd16+ cd7+ cd11b-	0.008563339	0.5078	0.1313	0.3782	3.8642
3 4 9 15 18	hla-dr- hla-dq- cd16+ cd11b- cd2+	0.004543777	0.3677	0.0629	0.2848	4.4341
3 6 8 9 14	hla-dr- cd26- cd45ra+ cd16+ cd71-	0.021311217	0.6184	0.2288	0.2886	2.2559
3 6 8 9 15	cd11b-	0.004204857	0.5169	0.1674	0.3595	3.2837
3 6 9 15 18	hla-dr- cd26- cd16+ cd11b- cd2+	0.005771116	0.2953	0.0682	0.2381	4.8874
3 8 9 14 15	hla-dr- cd26- cd16+ cd11b- cd2+	0.005914381	0.4889	0.1723	0.2867	2.7223
3 9 10 15 18	hla-dr- cd16+ cd57+ cd11b+ cd2+	0.022671738	0.0058	0.0768	-0.0710	13.2924
3 9 13 15 18	hla-dr- cd16+ cd82i- cd11b+ cd2+	0.023644883	0.0048	0.0881	-0.0843	18.5881
3 9 14 15 18	hla-dr- cd16+ cd71- cd11b- cd2+	0.016401998	0.3048	0.0872	0.2175	3.4941
1 3 7 9	cd4+ hla-dr+ cd36- cd16+	0.021057656	0.0111	0.0035	0.0075	3.1320
1 3 9 13	cd4+ hla-dr+ cd16+ cd82i-	0.024873506	0.0115	0.0038	0.0078	3.0721
1 3 9 15	cd4+ hla-dr+ cd16+ cd11b-	0.000763318	0.0141	0.0017	0.0124	8.2809
2 3 13 14	cd8- hla-dr+ cd82i- cd71+	0.017962464	0.0210	0.0609	-0.0399	2.9030
3 4 8 10	hla-dr+ hla-dq- cd26- cd57+	0.006148503	0.0102	0.0034	0.0089	3.0390
3 4 7 10	hla-dr+ hla-dq- cd36- cd57+	0.018170558	0.0078	0.0017	0.0081	4.6818
3 4 9 13	hla-dr+ hla-dq- cd16+ cd82i+	0.023279293	0.0049	0.0006	0.0043	8.0229

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3 4 10 18	hla-dr+ hla-dq- cd57+ cd2-	0.010408324	0.0089	0.0014	0.0056	5.1069
3 4 12 13	hla-dr+ hla-dq- cd7- cd82+	0.018389988	0.1594	0.0568	0.1028	2.8068
3 6 8 14	hla-dr+ cd26- cd45ra- cd71+	0.013552859	0.0095	0.0224	-0.0129	2.3605
3 6 9 14	hla-dr+ cd26- cd18+ cd71+	0.010378923	0.0076	0.0016	0.0058	4.5838
3 6 10 13	hla-dr+ cd26- cd57+ cd82+	0.008224371	0.0077	0.0024	0.0053	3.1931
3 7 14 16	hla-dr+ cd38+ cd71- cd38+	0.018413964	0.3539	0.2157	0.1382	1.8406
3 7 16 18	hla-dr+ cd38+ cd38+ cd2-	0.005860904	0.2550	0.1202	0.1349	2.1220
3 8 9 14	hla-dr+ cd45ra- cd18+ cd71+	0.016710778	0.0008	0.0001	0.0007	7.1178
3 8 13 14	hla-dr+ cd45ra- cd82- cd71+	0.011848945	0.0077	0.0288	-0.0181	3.4873
3 9 12 15	hla-dr+ cd18+ cd7+ cd11b-	0.002670709	0.0120	0.0015	0.0105	8.0658
3 9 14 18	hla-dr+ cd18+ cd71+ cd2-	0.011590973	0.0069	0.0019	0.0050	3.5878
3 9 15 18	hla-dr+ cd18+ cd11b- cd36+	0.003618084	0.0084	0.0000	0.0094	215.4480
3 12 13 18	hla-dr+ cd7- cd82+ cd36+	0.017289740	0.1148	0.0389	0.0747	2.8742
3 12 13 17	hla-dr+ cd7- cd82+ cd18-	0.007420254	0.2286	0.1263	0.1023	1.8100
3 12 15 16	hla-dr+ cd7- cd11b- cd36+	0.013106186	0.1413	0.0410	0.1003	3.4498
3 13 14 16	hla-dr+ cd82+ cd71- cd36+	0.004409924	0.2375	0.1287	0.1108	1.8753
3 13 16 18	hla-dr+ cd82+ cd36+ cd2-	0.002135838	0.1658	0.0579	0.1079	2.8840
3 13 17 18	hla-dr+ cd82+ cd19- cd2-	0.019218581	0.2872	0.1749	0.1222	1.8889
3 15 18 18	hla-dr+ cd11b- cd36+ cd2-	0.018930981	0.1946	0.0583	0.1383	3.3354
2 3 6 9 14	cd8- hla-dr+ cd26- cd18- cd71+	0.022963473	0.0227	0.0438	-0.0209	1.8189
2 3 9 13 14	cd8- hla-dr+ cd18- cd82- cd71+	0.010827838	0.0184	0.0600	-0.0435	3.8548
3 4 7 9 10	hla-dr+ hla-dq- cd38- cd18- cd57+	0.017486384	0.0079	0.0017	0.0083	4.7448
3 4 9 12 13	hla-dr+ hla-dq- cd16- cd7- cd82+	0.018887839	0.1834	0.0574	0.1080	2.8468
3 6 8 9 14	cd71+	0.011288839	0.0091	0.0225	-0.0134	2.4715
3 6 9 13 14	hla-dr+ cd26- cd18- cd82- cd71+	0.013228875	0.0136	0.0269	-0.0134	1.9978
3 7 9 14 16	hla-dr+ cd38+ cd18- cd71- cd38+	0.017998477	0.3603	0.2181	0.1421	1.6517
3 7 9 16 18	hla-dr+ cd38+ cd18- cd36+ cd2-	0.005251888	0.2801	0.1212	0.1388	2.1482
3 8 9 13 14	hla-dr+ cd45ra- cd18- cd82- cd71+	0.010365361	0.0073	0.0271	-0.0198	3.7277
3 9 10 13 14	hla-dr+ cd16- cd57- cd82- cd71+	0.022077938	0.0353	0.0787	-0.0414	2.1751
3 9 12 13 18	hla-dr+ cd16- cd7- cd82+ cd36+	0.017289898	0.1170	0.0405	0.0785	2.8987
3 9 12 13 17	hla-dr+ cd18- cd7- cd82+ cd19-	0.007378415	0.2340	0.1278	0.1062	1.8310
3 9 12 15 16	hla-dr+ cd16- cd7- cd11b- cd36+	0.015190888	0.1414	0.0413	0.1002	3.4275
3 9 13 14 18	hla-dr+ cd18- cd82+ cd71+ cd36-	0.023365941	0.0218	0.0363	-0.0145	1.8844
3 9 13 14 16	hla-dr+ cd18- cd82+ cd71- cd36+	0.004301036	0.2413	0.1281	0.1132	1.8840
3 9 13 16 18	hla-dr+ cd18- cd82+ cd36+ cd2-	0.001918288	0.1691	0.0583	0.1108	2.9019
3 9 13 17 18	hla-dr+ cd18- cd82+ cd19- cd2-	0.016790489	0.3023	0.1759	0.1265	1.7190
3 9 15 18 18	hla-dr+ cd18- cd11b- cd36+ cd2-	0.021886412	0.1947	0.0589	0.1359	3.3085
1 3 4 5 9	cd4+ hla-dr+ hla-dq- cd3- cd18+	0.021281551	0.1289	0.0389	0.0900	3.3112
1 3 4 9 13	cd4+ hla-dr+ hla-dq- cd18+	0.020781635	0.0855	0.0157	0.0499	4.1782
1 3 4 9 17	cd4+ hla-dr+ hla-dq- cd18+ cd19-	0.021504814	0.1631	0.0478	0.1183	3.4112
1 3 5 8 9	cd4+ hla-dr+ cd3+ cd45ra- cd18+	0.014439827	0.0280	0.0038	0.0243	7.4338
1 3 6 9 18	cd4- hla-dr+ cd26- cd18+ cd36-	0.022946238	0.2225	0.5968	-0.3733	2.8773
1 3 7 9 15	cd4+ hla-dr+ cd38+ cd18+ cd11b-	0.002071532	0.1188	0.0220	0.0950	5.3184
1 3 8 10 18	cd4- hla-dr+ cd16+ cd57- cd36-	0.018828143	0.1856	0.8189	-0.4344	3.3410
2 3 8 9 18	cd8- hla-dr+ cd45ra- cd18+ cd2+	0.014502942	0.0428	0.0061	0.0384	6.9321
2 3 9 12 16	cd8- hla-dr+ cd18+ cd7+ cd11b-	0.002143373	0.2801	0.0370	0.2432	7.5788
2 3 9 13 15	cd8- hla-dr+ cd18+ cd82+ cd11b-	0.007675771	0.2083	0.0144	0.1839	14.4727
2 3 9 15 18	cd8- hla-dr+ cd18+ cd11b- cd2+	0.020478189	0.1532	0.0198	0.1334	7.7337
3 4 5 8 9	hla-dr+ hla-dq- cd3+ cd45ra- cd18+	0.018029725	0.0178	0.0008	0.0173	31.7121
3 5 7 9 16	hla-dr+ cd3- cd38- cd18+ cd36+	0.016878443	0.1531	0.0287	0.1234	5.1544
3 5 9 13 15	hla-dr+ cd3- cd18+ cd82+ cd11b-	0.018287587	0.1860	0.0116	0.1744	16.0015

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same visual field. Note that many of the cells carry abnormal protein clusters. Fig. 9 gives all optical sections through each of the 3 protein signals indicated by an arrow in Fig. 10a). Fig. 10b) gives the most significant 3D cell surface protein clusters in ALS and Fig. 10a) gives the 3D visualisation of all the cells shown as an optical section illustrated in Fig. 8. As indicated by the different colours (Fig. 10a) each protein has a unique distribution pattern over the cell surface of this individual MNC. Some areas of this MNC show a substantial overlap of the protein signals whilst other parts of the cell surface are characterised by one of these proteins alone. Note that each protein pattern is demanded as rendered volume on the cell surface of each MNC. Overlapping sites of the proteins are not visualized. Fig. 11 gives another cycle of the cell surface protein cluster shown in Fig. 10a.

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MELTEC

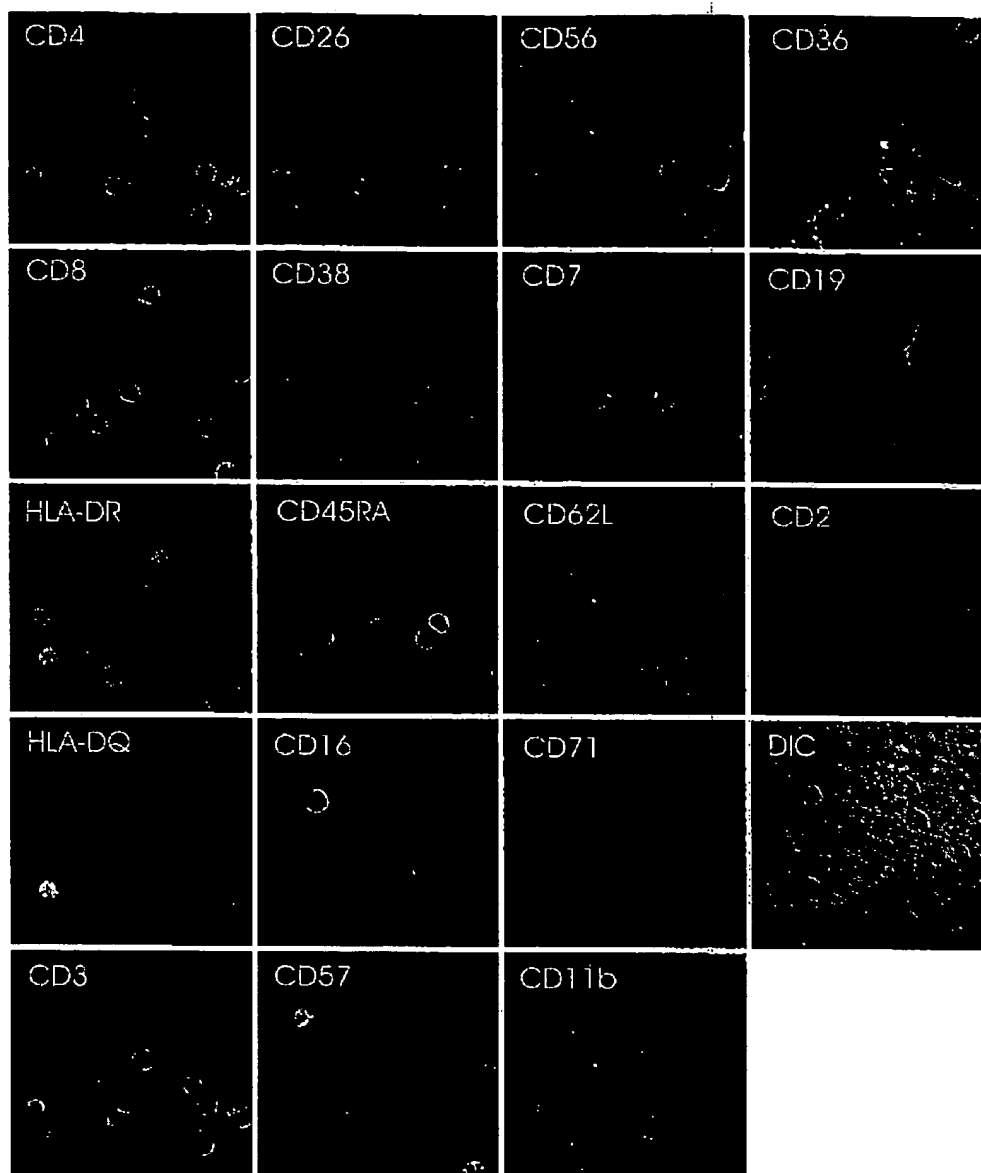


Fig. 8

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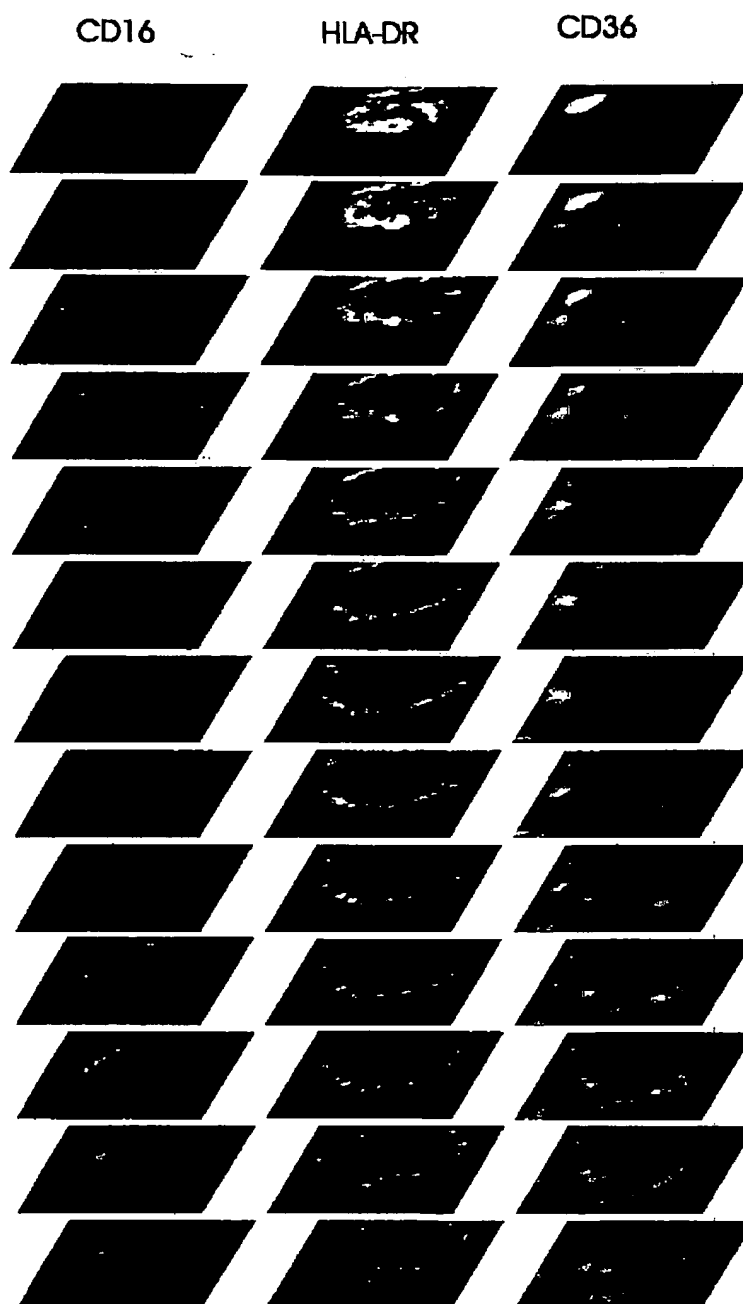
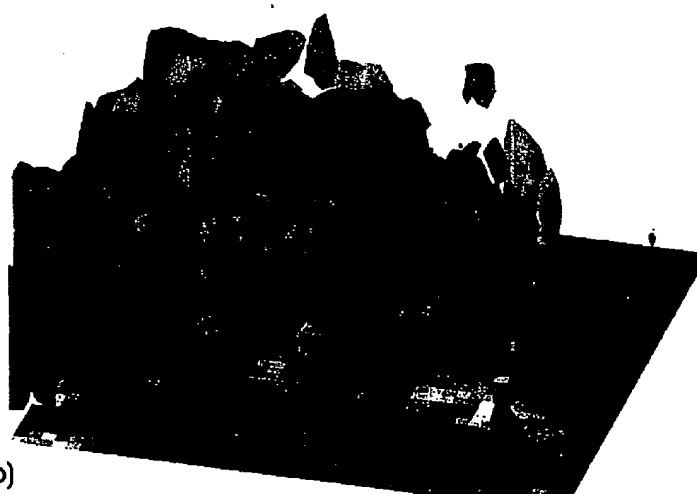


Fig. 9

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a)



b)

■	CD16
■	CD26
■	CD36
■	CD3
■	CD45RA
■	CD4
■	CD56
■	CD57
■	CD71
■	CD8
■	HLA-DQ
■	HLA-DR
■	CD11b
■	CD62L
■	CD7
■	CD2
■	CD19
■	CD38

Fig. 10

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Fig 11

Together the data indicate, that both in normal individuals and in ALS there are several CPP motifs which contain CD16 as the "leading protein", but the motifs are inherently different from each other:

In normal individuals CD 16 co-clusters with CD 11b/CD2 or hladr; if CD 16 co-clusters with hladr the following molecules are always absent: CD11b, CD62l, CD36 and CD 4. This indicates that this invers correlation of cell surface receptors is important for the normal state of MNC.

In contrast, in ALS-CD16, which co-clusters with hladr does strictly also co-cluster with CD36 or CD36 + CD 62l, but never with CD11b. In addition CD16 in ALS does also co-cluster with CD45ra. These clusters strictly exclude hladr and CD11b.

Together these ALS-specific cell surface protein clusters indicate presence of abnormal cell surface differentiation events. On the basis of the given high statistical significance these data represent a valid peripheral biomarker for ALS and basis for CD16-oriented modification therapy.

Technology overview

Imaging-Cycler MELK: Set up

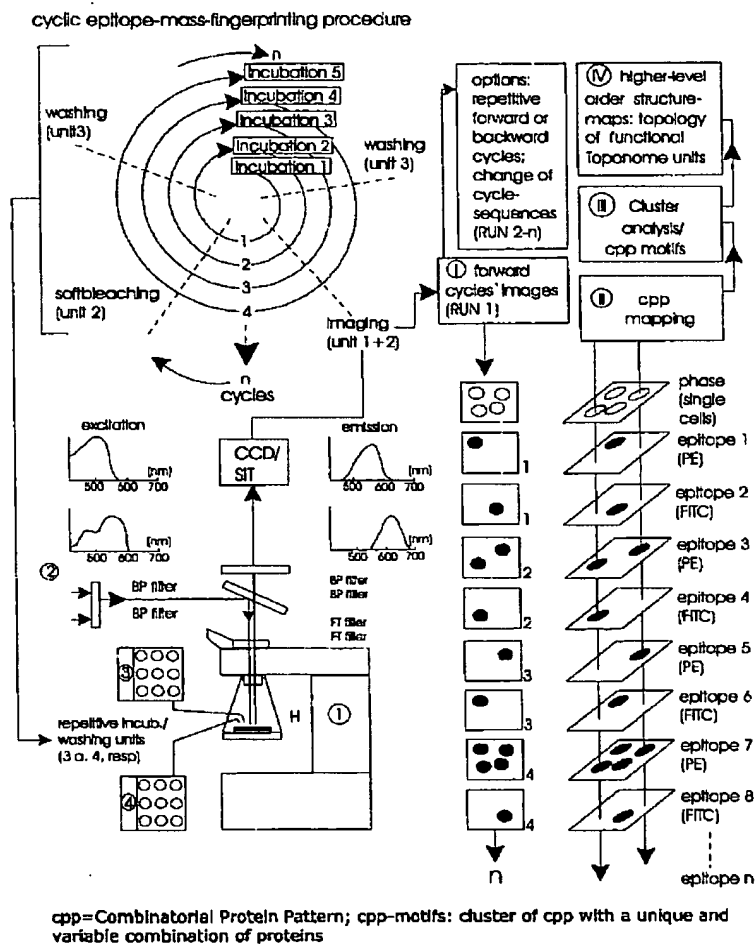


Fig. 1

Toponomic Fingerprinting

Automated Workflow

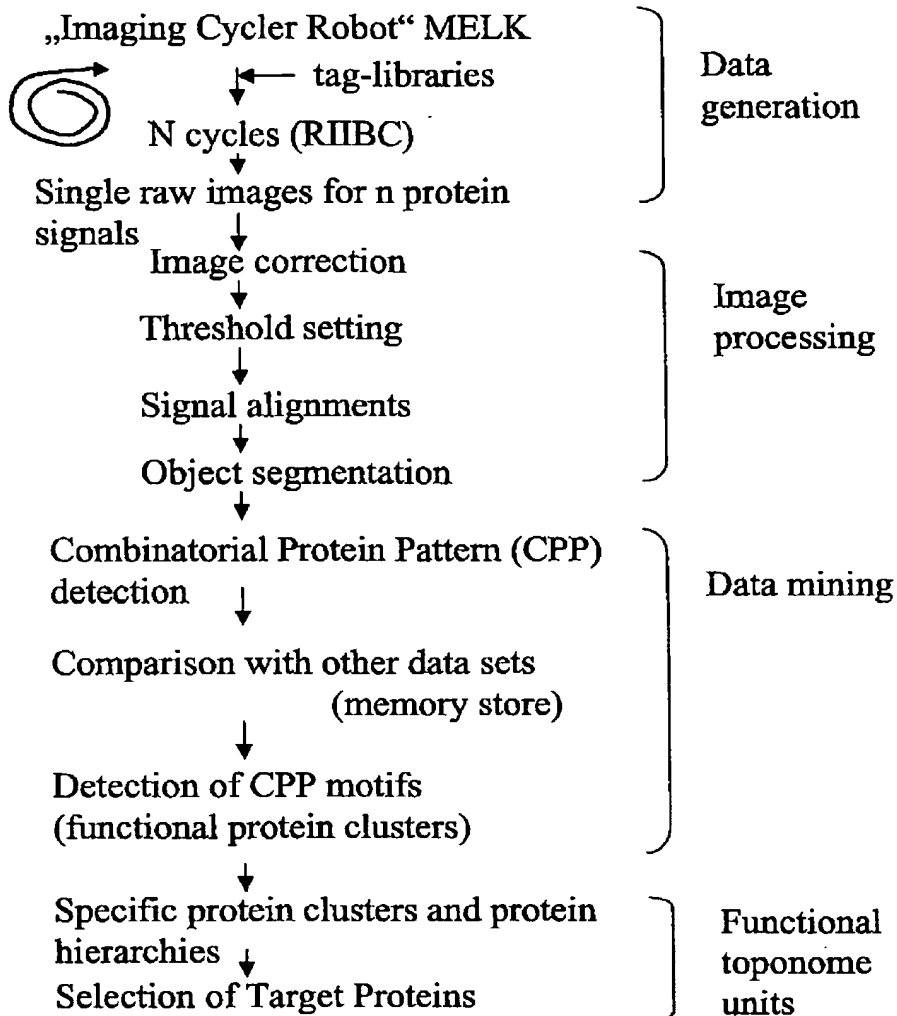


Fig. 2

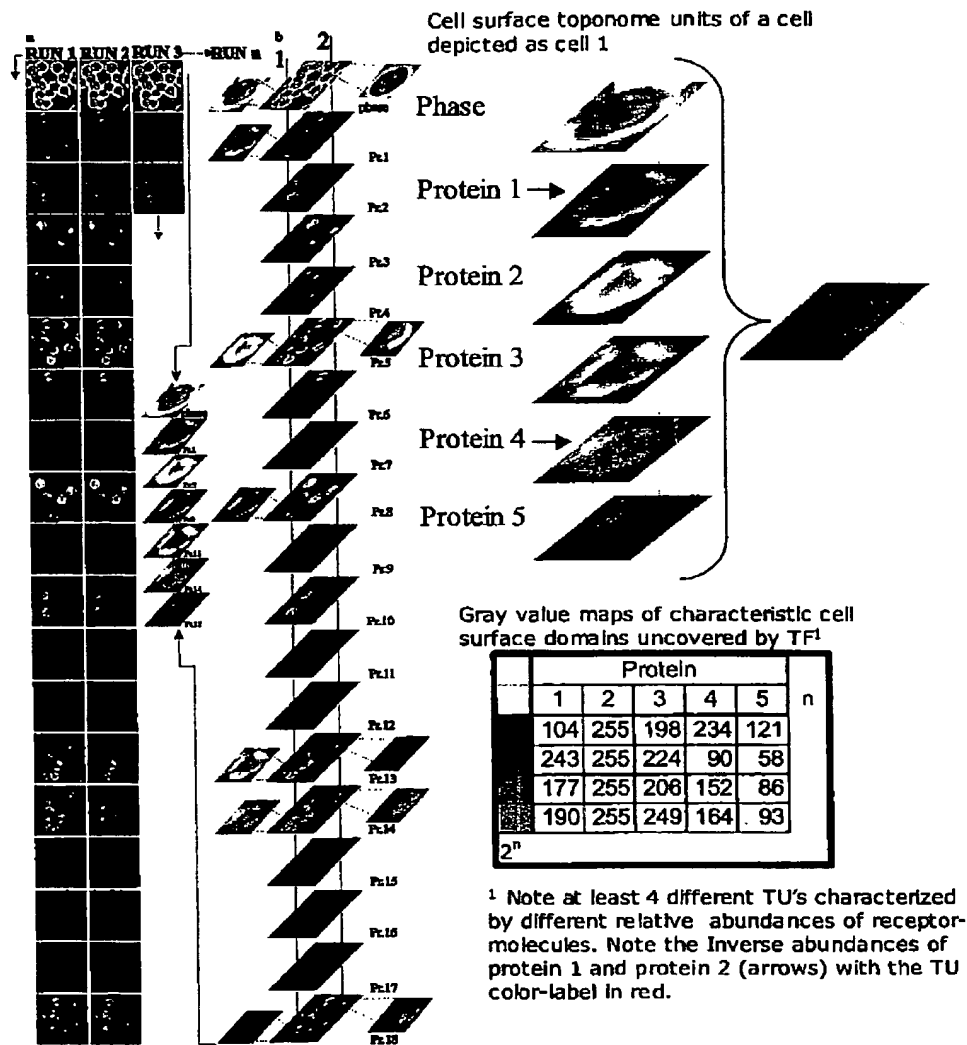


Fig. 3

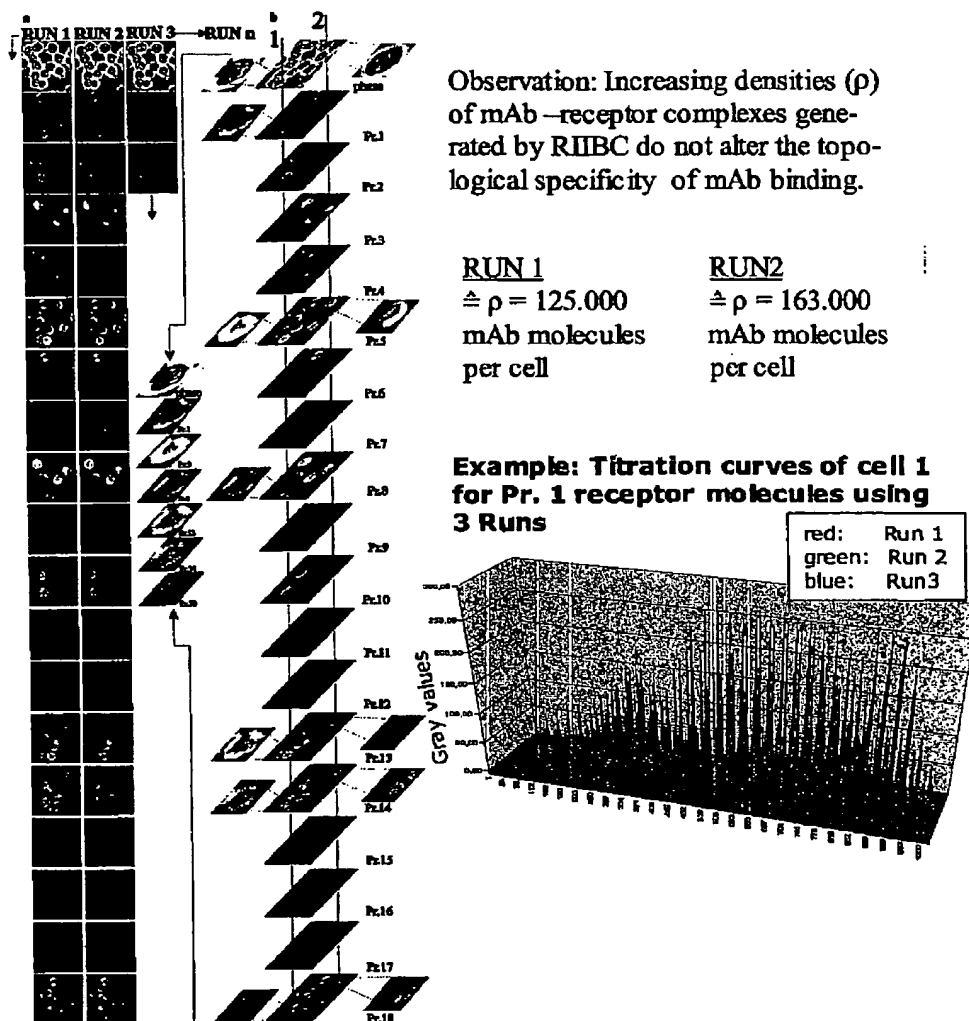


Fig. 4

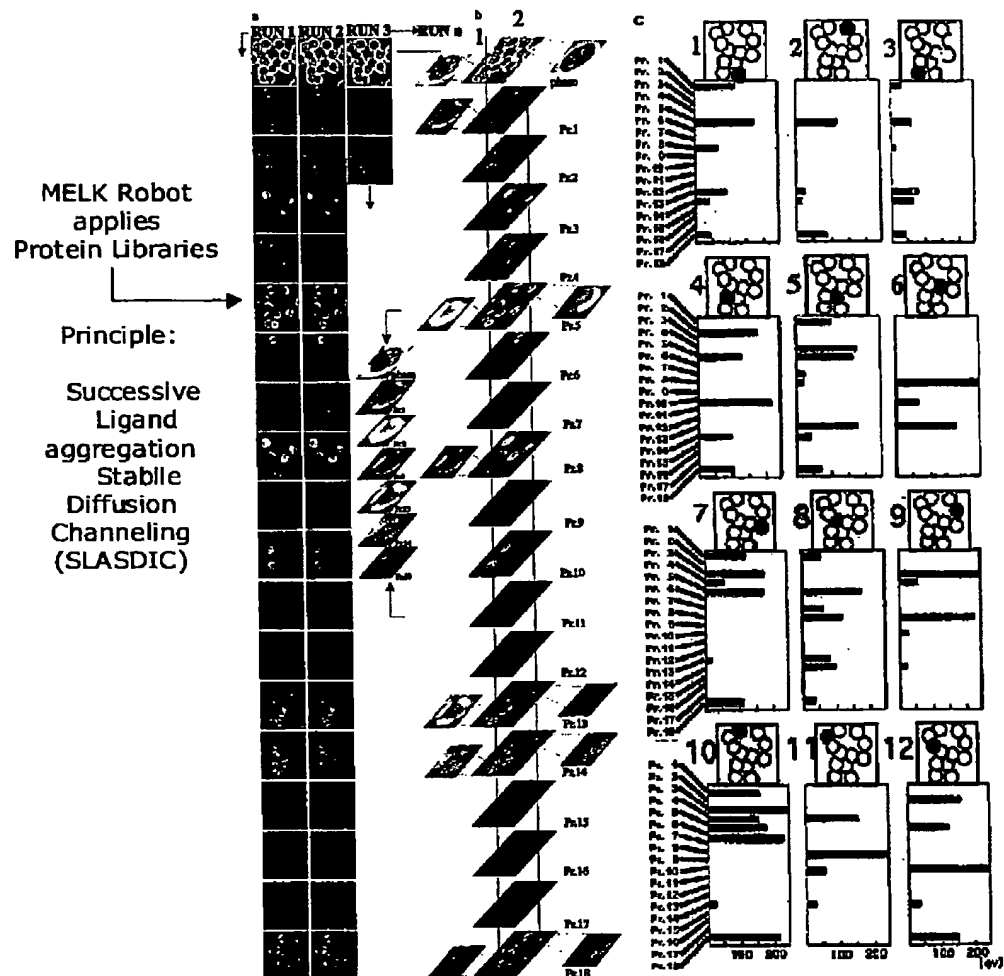
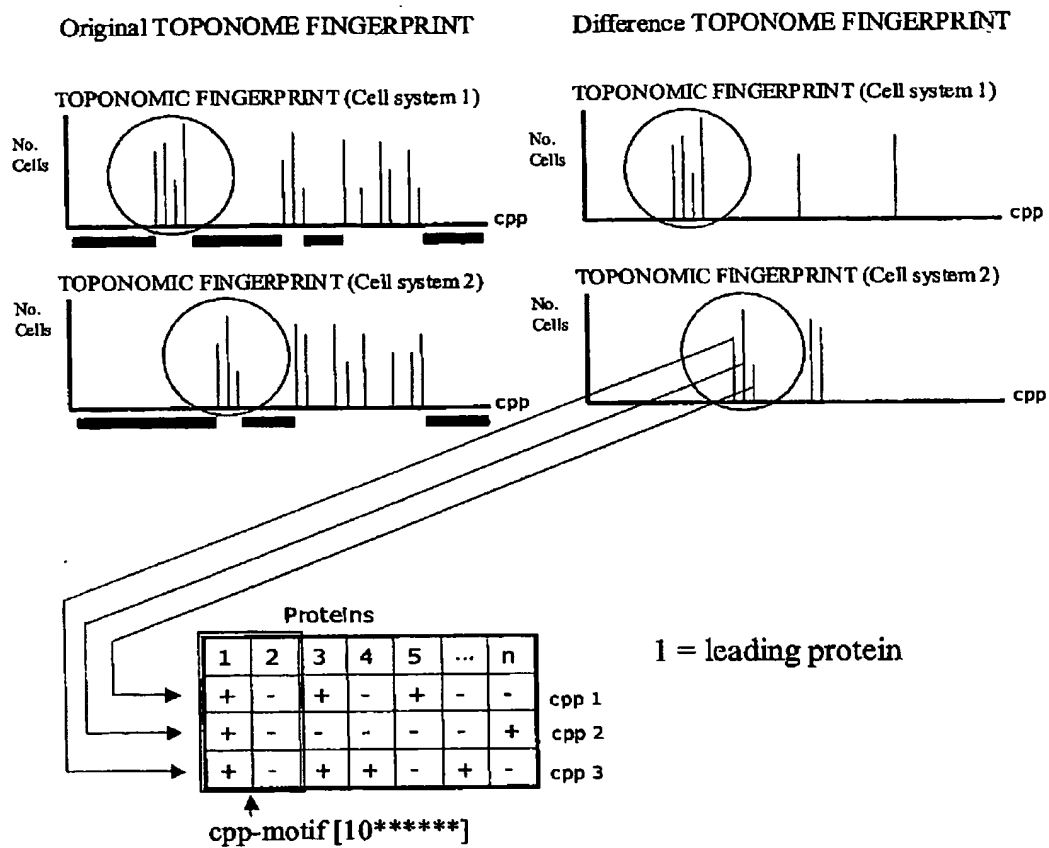
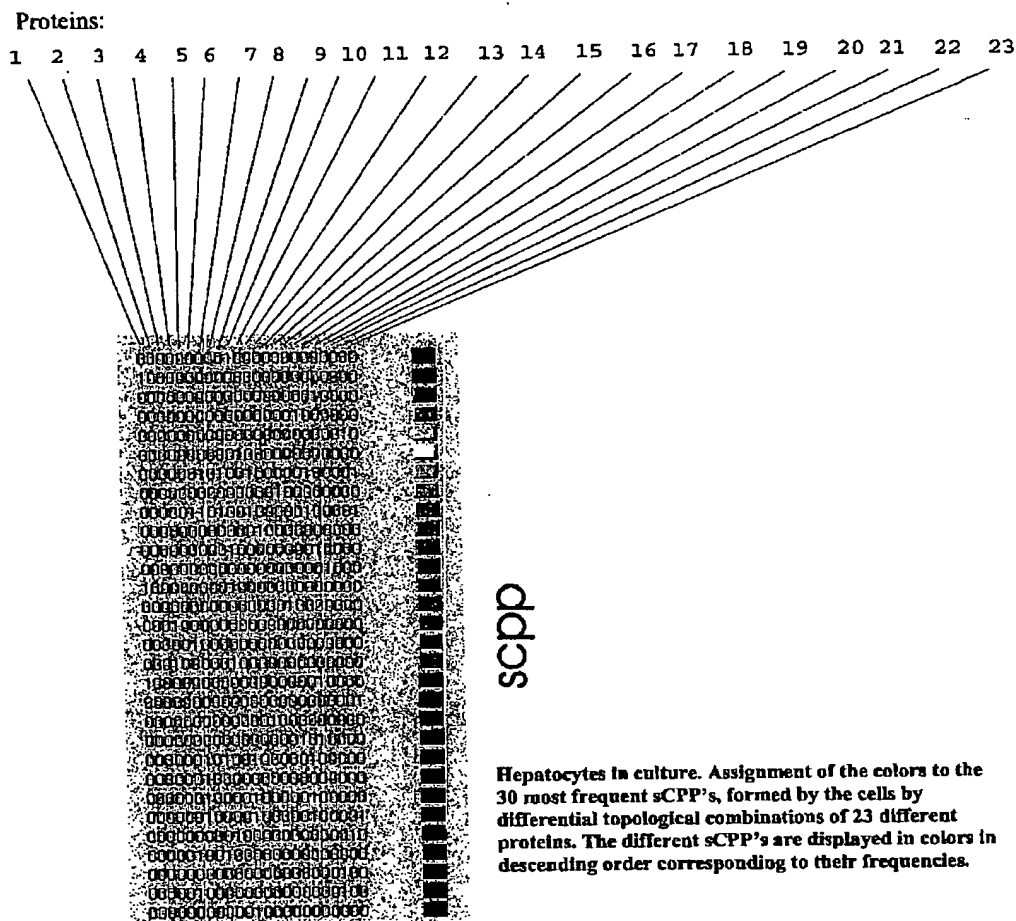


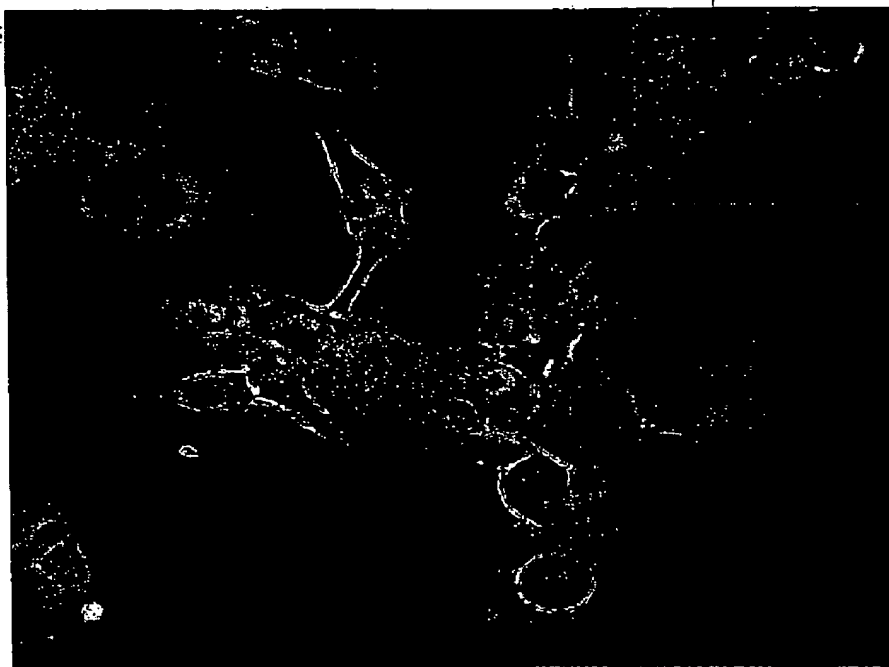
Fig. 5

**Fig. 6**

Biological example I

Two-dimensional toponome fingerprinting of Hepatocytes in Culture

**Fig. 7**

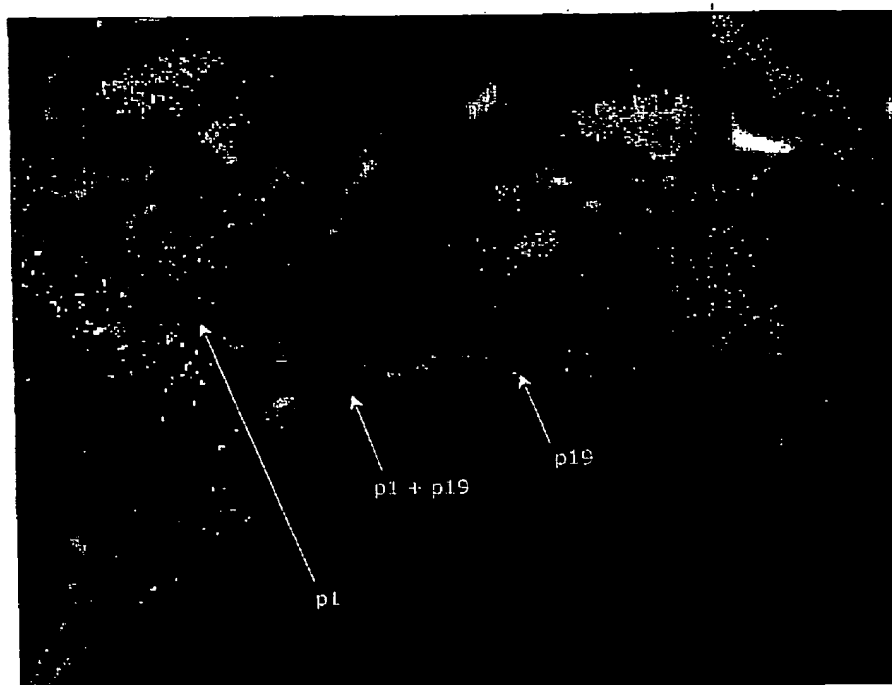


Hepatocytes in
culture, control.



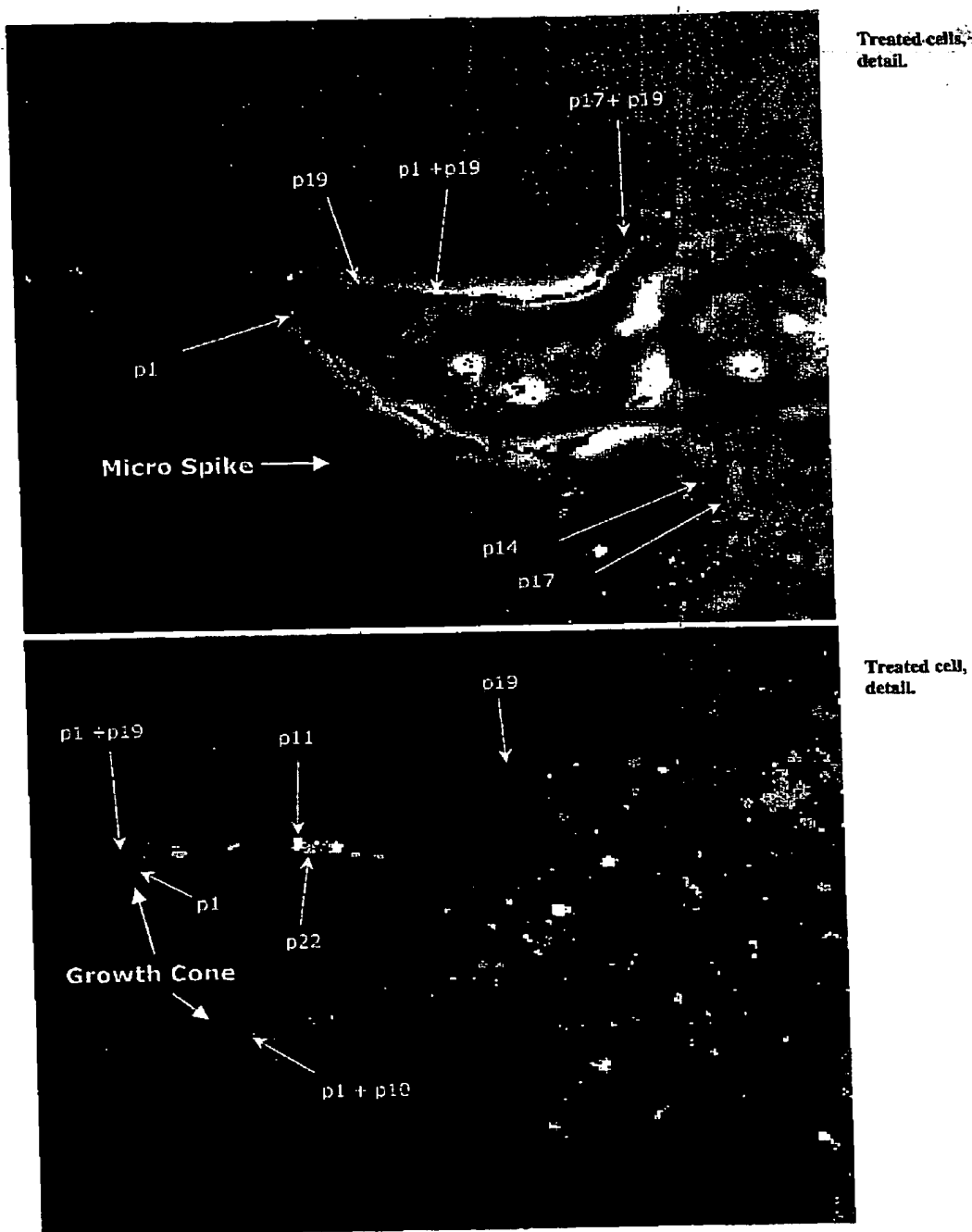
Hepatocytes in
culture, treated
cells.

Fig. 8



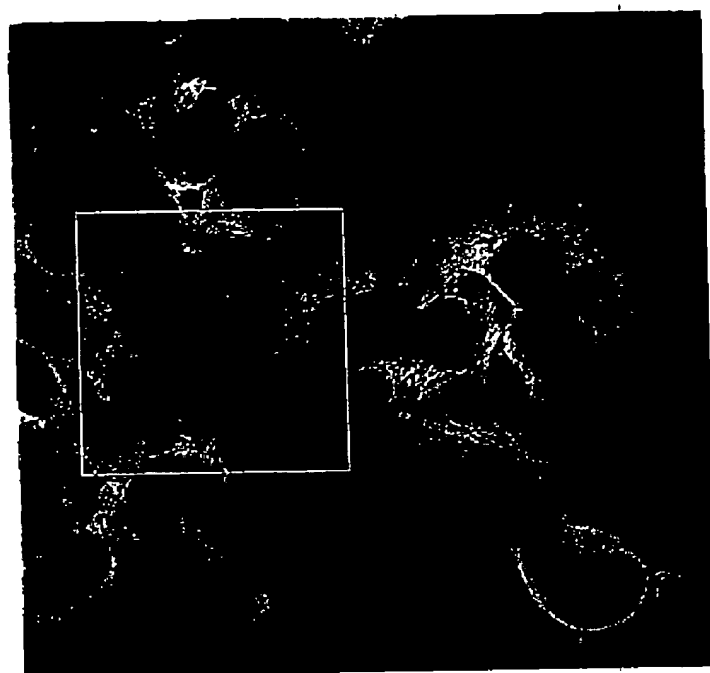
Control cell,
Detail.

Fig. 9

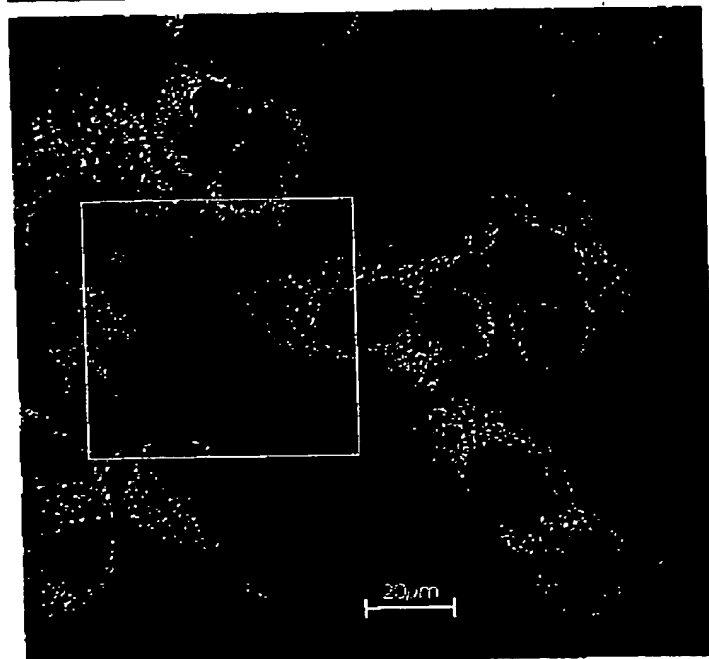
**Fig. 10**

Biological Example II

**Threedimensional mapping of subcellular
toponome "landmarks" of
Hepatocytes in culture
as a
basis for mapping-in functional
protein complexes**

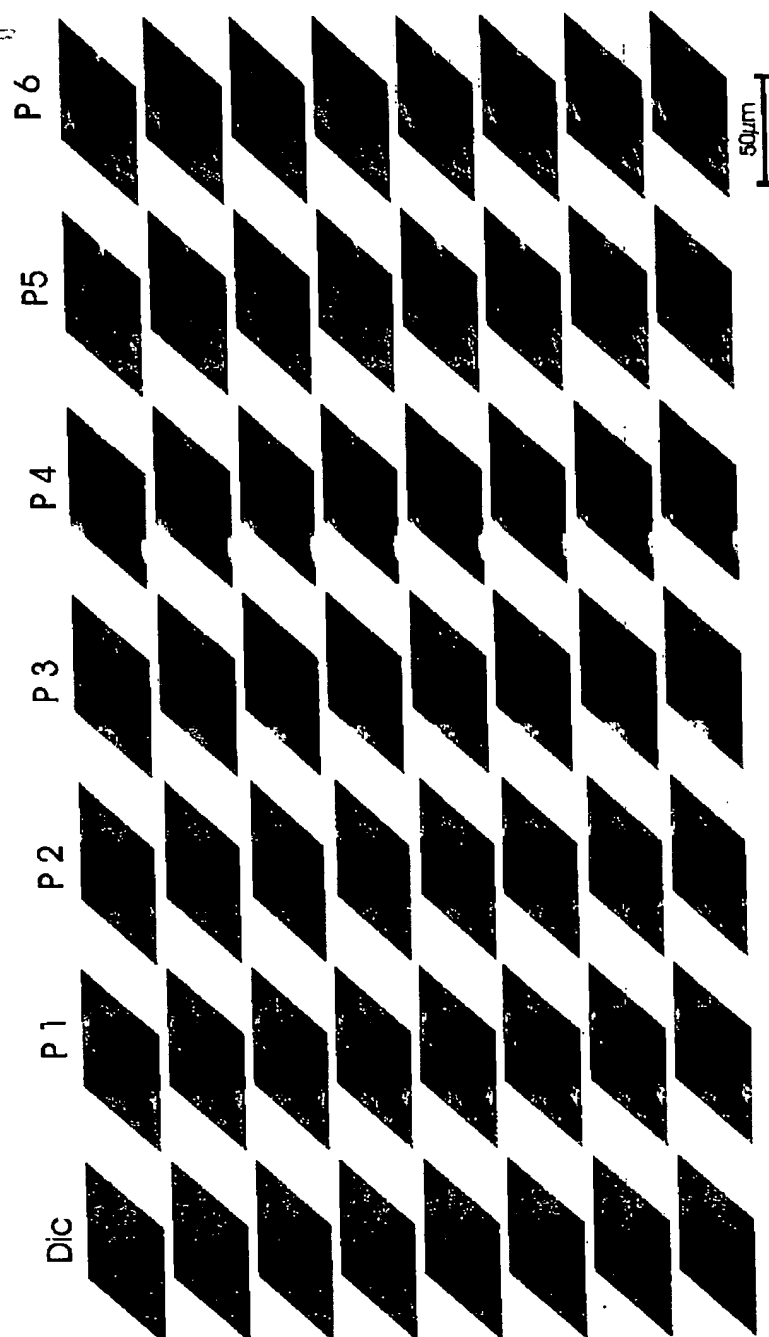


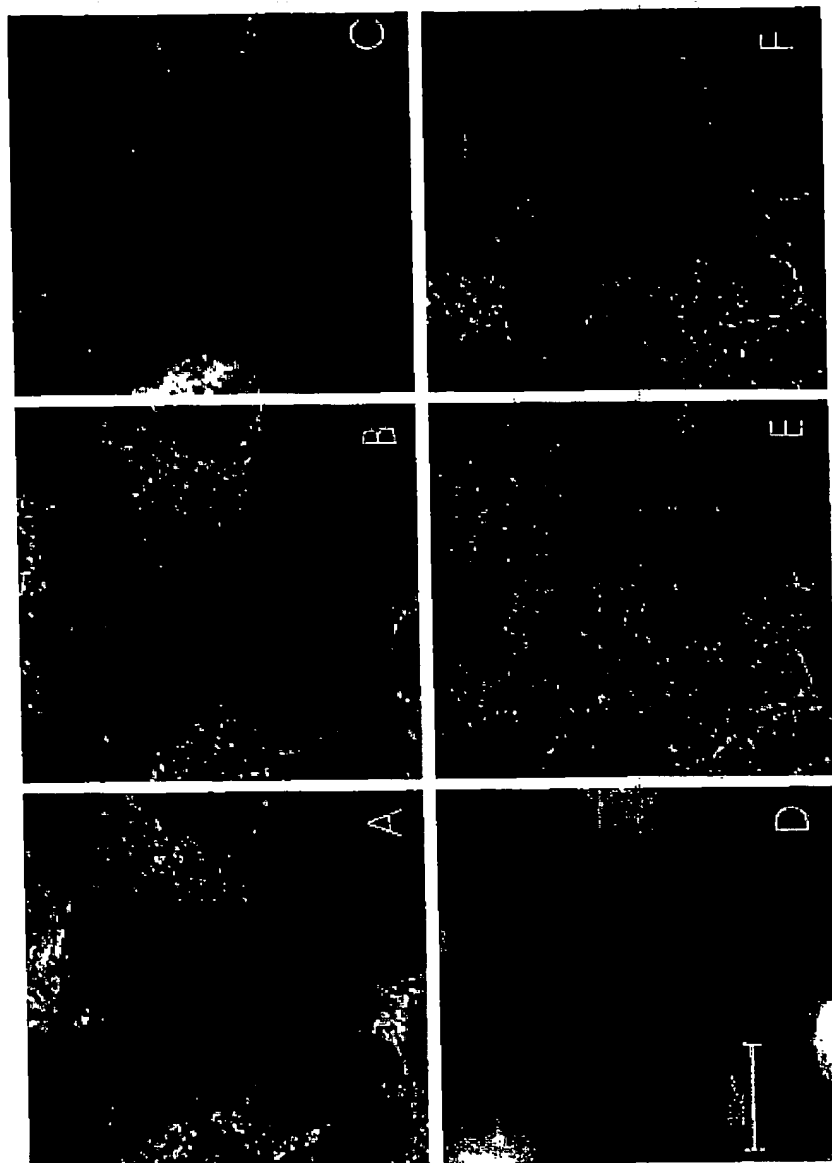
P 1



P 2

Fig. 11

**Fig. 12**

**Fig. 13**

- P 1 - Cytokeratin marker
- P 2 - Mitochondrial marker
- P 3 - Golgi-trans Golgi marker
- P 4 - DNA marker
- P 5 - Trans Golgi vesicle marker
- P 6 - Integrin complex

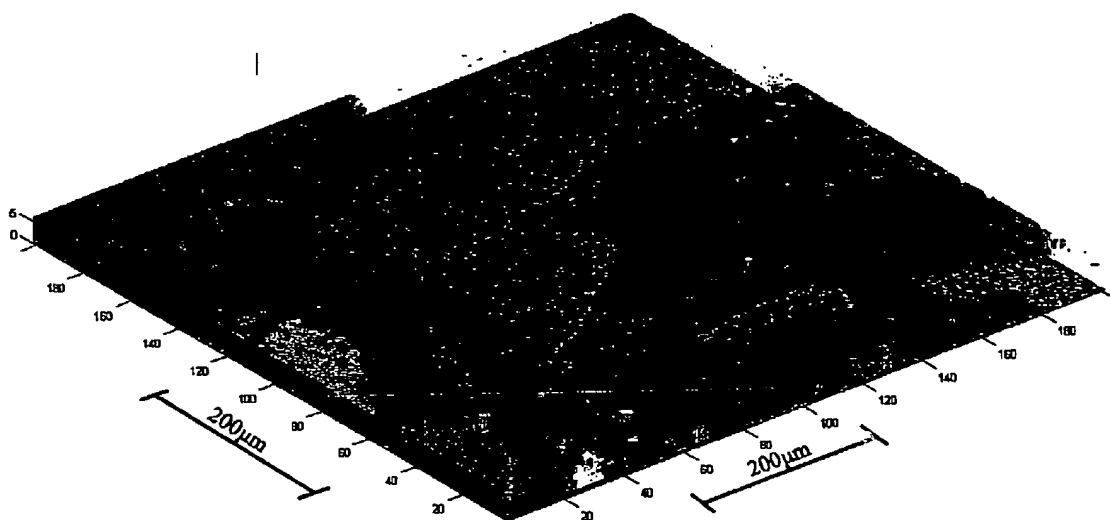


Fig. 14



Fig. 15

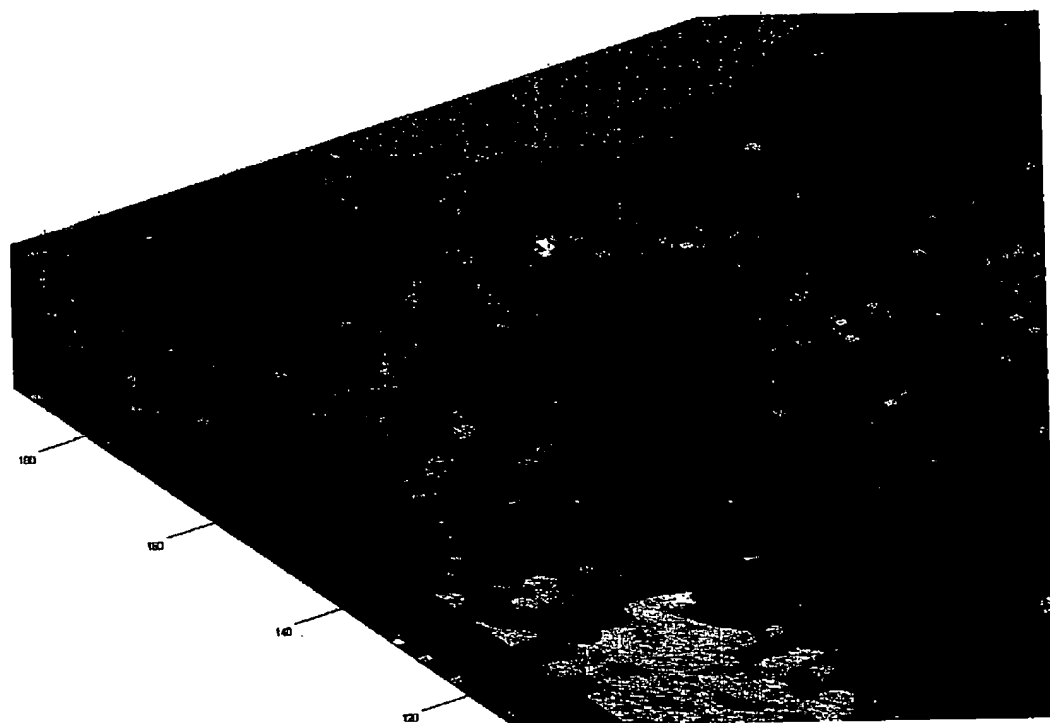
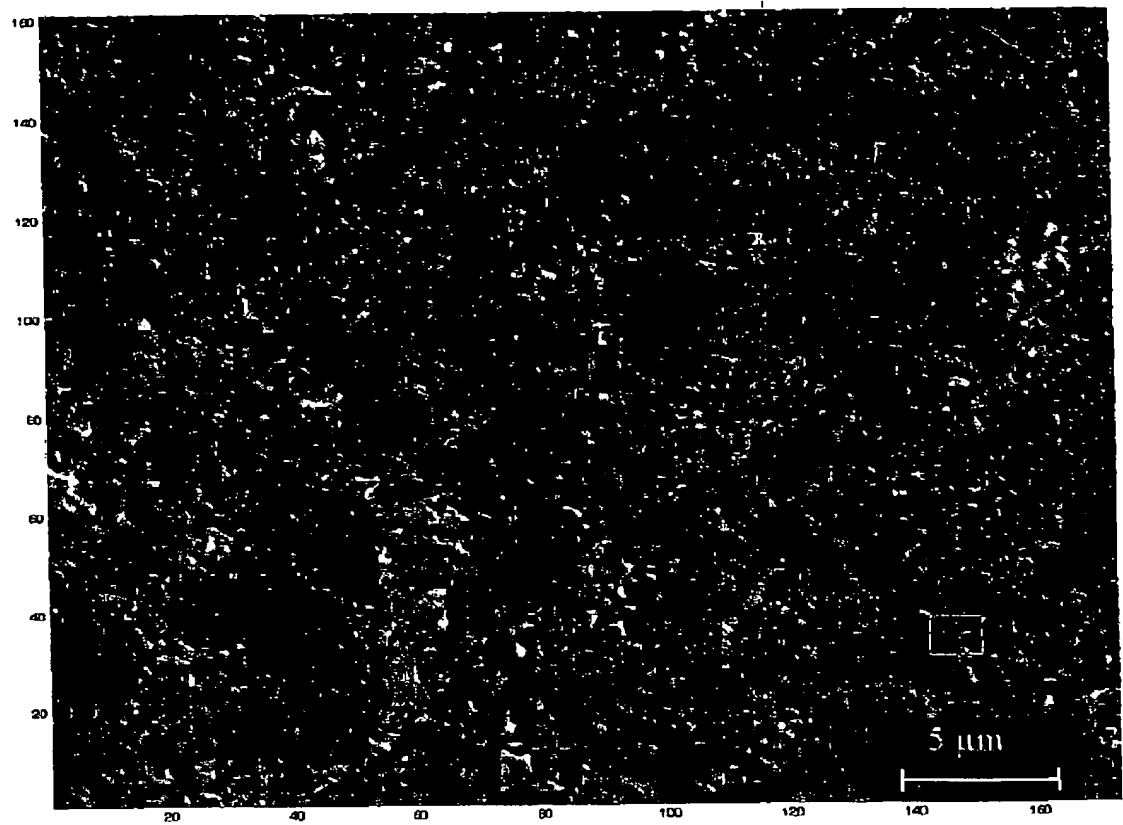


Fig. 16

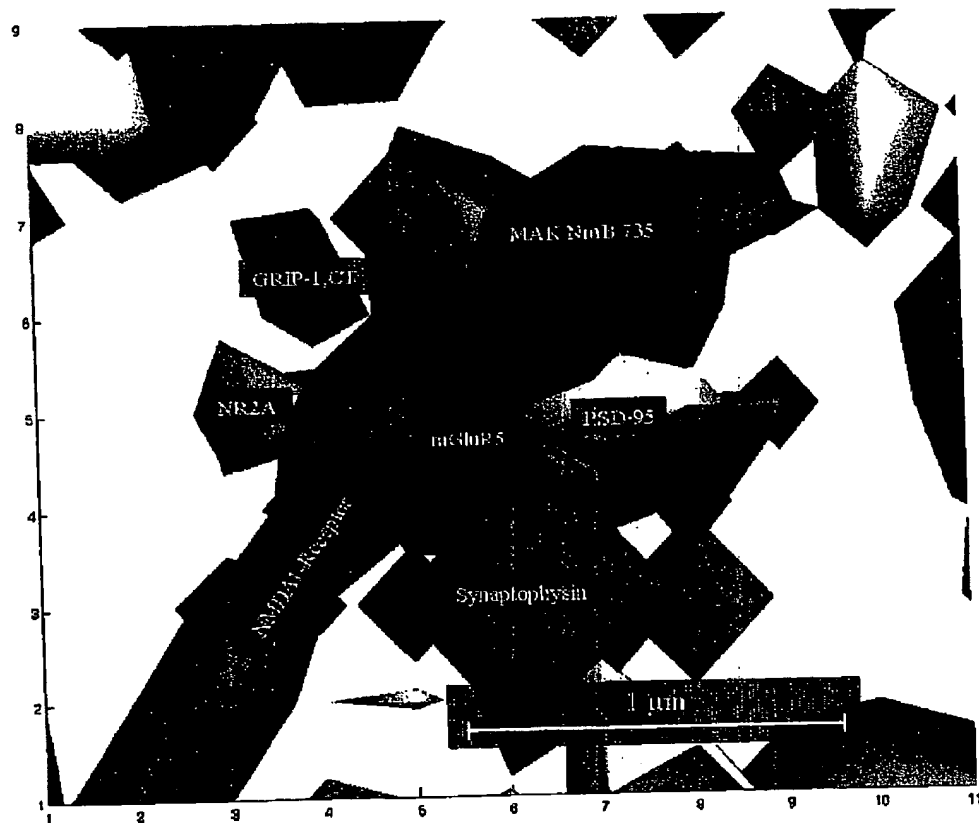
Biological example III

**Threedimensional toponome fingerprinting
to detect
protein-complexes in tissue sections
of
the Spinal Cord**



- GluR2/3
- mGluR5
- GRIP-1,CT
- MAK NmB 735
- NR2A
- NMDA1
- Propidium Iodid
- PSD-95
- Synaptophysin

Fig. 17

**Fig. 18**

DECLARATION APPENDIX B: REFERENCES

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ALS Web page, 2006

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Takai, T., 2000

Hogarth, P.M., 2002

Sautes-Fridman et al., 2003



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1: J Chromatogr B Biomed Appl. 1994 Dec 9;662(2):197-207.

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Recombinant soluble Fc gamma receptors: production, purification and biological activities.

Sautes C, Galinha A, Bouchard C, Mazieres N, Spagnoli R, Fridman WH.

INSERM UNITE 255, Institut Curie, Paris, France.

Soluble forms of low affinity receptors for the Fc portion of IgG circulate in body fluids and regulate immune functions. We describe the transfection, production and purification techniques which allow the preparation, at a laboratory scale, of milligram amounts of glycosylated recombinant mouse and human soluble Fc gamma receptors. These recombinant products bind IgG and are biologically active on immune responses, like their normal counterparts.

PMID: 7719476 [PubMed - indexed for MEDLINE]

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Annual Review of Immunology

Vol. 19: 275-290 (Volume publication date April 2001)
(doi:10.1146/annurev.immunol.19.1.275)

IGG FC RECEPTORS

Jeffrey V. Ravetch and Silvia Bolland

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Since the description of the first mouse knockout for an IgG Fc receptor seven years ago, considerable progress has been made in defining the in vivo functions of these receptors in diverse biological systems. The role of activating FcγRs in providing a critical link between ligands and effector cells in type II and type III inflammation is now well established and has led to a fundamental revision of the significance of these receptors in initiating cellular responses in host defense, in determining the efficacy of therapeutic antibodies, and in pathological autoimmune conditions. Considerable progress has been made in the last two years on the in vivo regulation of these responses, through the appreciation of the importance of balancing activation responses with inhibitory signaling. The inhibitory FcR functions in the maintenance of peripheral tolerance, in regulating the threshold of activation responses, and ultimately in terminating IgG mediated effector stimulation. The consequences of deleting the inhibitory arm of this system are thus manifested in both the afferent and efferent immune responses. The hyperresponsive state that results leads to greatly magnified effector responses by cytotoxic antibodies and immune complexes and can culminate in autoimmunity and autoimmune disease when modified by environmental or genetic factors. FcγRs offer a paradigm for the biological significance of balancing activation and inhibitory signaling in the expanding family of activation/inhibitory receptor pairs found in the immune system.

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Hongwei Gao, Thomas Neff, Peter A. Ward

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1: Annu Rev Immunol. 2000;18:709-37.

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Regulation of antibody responses via antibodies, complement, and Fc receptors.

Heyman B.

Department of Genetics and Pathology, Uppsala University, Sweden.
birgitta.heyman@genpat.uu.se

Antibodies can completely suppress or enhance the antibody response to their specific antigen by several hundredfold. Immunoglobulin M (IgM) enhances antibody responses via the complement system, and complement activation by IgM probably starts the chain of events leading to antibody responses to suboptimal antigen doses. IgG can enhance primary antibody responses in the absence of the complement system and seems to be dependent on Fc receptors for IgG (FcγRs). IgE enhances antibody responses via the low-affinity receptor for IgE (FcεRI/CD23). The precise effector mechanisms that cause enhancement are not known, but direct B-cell signaling, antigen presentation, and increased follicular localization are all possibilities. IgG, IgE, and IgM may also suppress antibody responses when used in certain immunization regimes, and it seems reasonable that an important mechanism behind suppression is the masking of antigenic epitopes by antibodies. In addition, FcγRIIB, which contains a cytoplasmic inhibitory motif, acts as a negative regulator of antibody responses. This receptor, however, may prevent the antibody responses from exceeding a certain level rather than causing complete suppression.

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Nature Reviews Immunology 2, 580-592 (2002); doi:10.1038/nri856

ROLES OF FC RECEPTORS IN AUTOIMMUNITY

Toshiyuki Takai about the author

Abstract

The receptors for the Fc of immunoglobulins, Fc receptors (FcRs), link the humoral and cellular branches of the immune system, and they have important functions in the activation and down-modulation of immune responses. Balanced signalling through activating and inhibitory FcRs regulates the activity of various cells in the immune system. Recent work in animal models indicates that the development of many human autoimmune diseases might be caused by impairment of the FcR regulatory system. This review provides an overview of the mechanisms of FcR-based immune regulation and describes how autoimmune disease might result from its dysfunction.

Summary

- Fc receptors for immunoglobulin G (FcγRs) link humoral and cellular immunity by binding antigen-IgG immune complexes and internalizing the complexes for efficient antigen presentation. FcγRs comprise many activating-type receptors and a unique inhibitory receptor, FcγRIIB.
- FcγRIIB is a crucial element of peripheral tolerance. Its absence renders B cells and effector cells hyperresponsive to autoantigens.
- In the absence of the Fc-receptor common γ-chain, a pivotal adaptor for activating signalling, mouse models do not develop spontaneous or induced autoimmune disease due to the lack of activation of effector cells, such as macrophages.
- FcγRIIB-deficient mice have enhanced responses in some autoimmune disease models. Spontaneous onset of disease is sometimes observed in the mutant mice.
- Enhanced antigen presentation by Fc receptors on dendritic cells can be an important step in the development of some T-cell-mediated autoimmune diseases.
- Polymorphisms in the ectodomains of human FcγRs are risk factors for autoimmune diseases.
- Some polymorphisms are found in the human *FcγRIIB* gene, but information on their relation to autoimmune diseases is still required.
- The dynamics of the intimate collaboration between activating and inhibitory FcγRs might determine the balance between tolerance and autoimmunity.

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1: Curr Opin Immunol. 2002 Dec;14(6):798-802.

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Fc receptors are major mediators of antibody based inflammation in autoimmunity.

Hogarth PM.

Helen McPherson-Smith Laboratory, Austin Research Institute, Heidelberg, VIC, Australia. pm.hogarth@ari.unimelb.edu.au

There is now renewed interest in the role of antibodies in autoimmunity. Recent compelling evidence indicates that autoantibodies and the effector mechanisms they induce, for example, Fc receptor activation of leukocytes and/or the complement cascade, are central players in the development of autoimmunity, by perpetuating inflammation and perhaps even regulating the process itself. Of increasing interest are Fc receptors, which have been more closely investigated in the past decade using recombinant proteins, gene deficient mice and mouse models of human disease. These analyses point towards major roles of Fc receptors in antibody hypersensitivity reactions and by extension autoimmune disease, and they reveal opportunities in the development of novel therapeutic approaches in the treatment of autoimmune diseases.

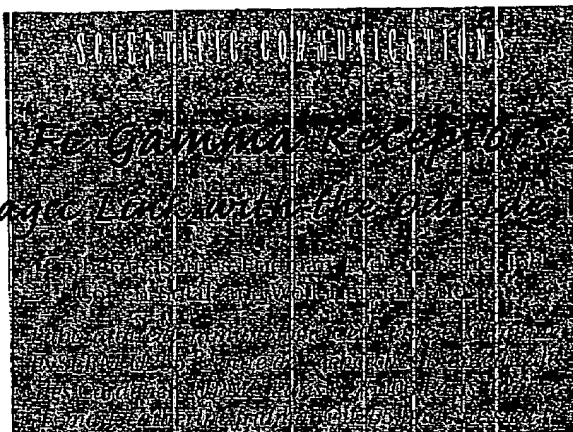
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A Magic Lock with the Outside World



Almost every cell of the immune system expresses receptors that are specific for the Fc region of Ig isotypes. The Fc receptors for IgG (FcγR) play a critical role in immunity by linking the IgG antibody mediated responses with cellular effector and regulatory functions of the immune system.¹ Depending on their cytoplasmic region and/or their associated chains, FcγR display both coordinate and opposing roles in immune responses (Figure 1). The activating receptors contain an Immunoreceptor Tyrosine-based Activation Motif (ITAM) in their cytoplasmic region or in their associated signal transducing units.^{2,3} They initiate inflammatory, cytolytic and phagocytic activities of immune effector cells.⁴ The inhibitory receptors contain an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in their cytoplasmic tail.⁵ Upon cross-linking with ITAM-containing receptors, they down regulate responses. For instance, the inhibitory receptors negatively

regulate Antigen-specific proliferation and differentiation of B cells, IgE-triggered mediator release by mast cells and internalization of IgG-immune complexes by macrophages.⁶ Three classes of FcγR exist. FcγRI are high affinity receptors (Kd = 10⁻⁸ M for monomeric IgG) whereas FcγRII and FcγRIII exhibit low affinity for monomeric IgG, with Kd for monomeric IgG

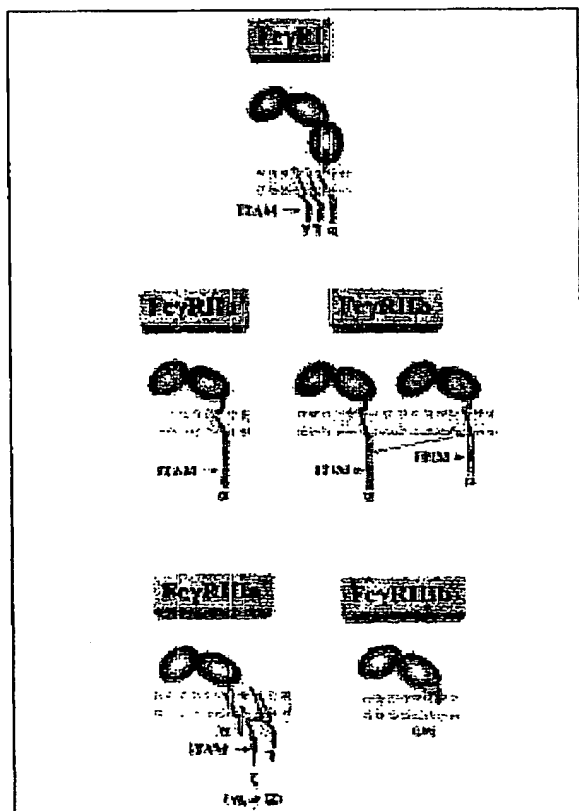


Figure 1

	B-Lymphocytes	Dendritic cells*	Macrophages Monocytes	NK-cells	Neutrophils	Mast cells
FcγRI						
FcγRIIa						
FcγRIIb						
FcγRIIB						
FcγRIIB						
Remarks:					FcγRIIb induced by IFNγ	

(* FcγR expression depends on their activation status. Some FcγR are also expressed on Langerhans cells, Eosinophils, Platelets, Endothelial cells, Mesangial cells, Metastatic melanoma)

Table 1

All cell types of the Immune System express functional FcγR

ranging from 10⁻⁵ to 10⁻⁷ M. The receptors are composed of two extracellular domains (FcγRII and FcγRIII) to three extracellular domains (FcγRI) and belong to the Ig-super family. Whereas FcγRI, FcγRIIa and FcγRIII are activating receptors, FcγRIIb1 and FcγRIIb2 are inhibitory receptors generated by alternative splicing. With the exception of NK cells and B cells that exclusively express FcγRIIa and FcγRIIb respectively, most cell types express both activating and inhibitory receptors (Table 1).⁴ The cellular response depends on the ratio between activating and inhibitory receptors, and hence on the cytokine environment, since Th1 and Th2 cytokines up-regulate the expression of activating and inhibitory receptors respectively. FcγRIIb is the unique FcγR anchored to the plasma membrane via a C-terminus-linked GPI moiety.⁷ Present exclusively on neutrophils, it plays a predominant role in binding of immune complexes, and its aggregation activates phagocytosis, degranulation, and the respiratory burst leading to destruction of opsonized pathogens.^{8,9} Activation of neutrophils leads to secretion of a proteolytically cleaved soluble form of the receptor corresponding to its two extracellular domains.¹⁰ Soluble FcγRIIb exerts regulatory functions by competitive inhibition of FcγR-dependent effector functions and via binding to the complement receptor CR3,¹¹ leading to production of inflammatory mediators.

SCIENTIFIC CORRESPONDENCE

Recent progress in the FcγR field led to the concept that FcγR control the balance between autoimmunity and tolerance in the periphery. In addition, their opposing roles in antibody-dependent effector cell responses makes them important partners for antibody-based therapies.

How Fcγ receptors bind IgG

The Fc region is separated from the antigen binding parts of the IgG molecule by a flexible hinge region and forms two structural domains, the CH2 and CH3 domains. Cellular and structural approaches have shown that the lower hinge region contains the major binding site for FcγR. It is established that cross-linking of FcγR membrane molecules is a prerequisite to IgG-mediated cell activation. Since the Fc portion is composed of two identical polypeptide chains that are related to each other by a two-fold axis, each IgG molecule may potentially bind up to two FcγR and initiate cellular responses even in the absence of multivalent antigen. However, stoichiometry of the interaction of soluble FcγRII or III with IgG is 1:1, in solution. Studies by NMR have suggested that a rearrangement occurs in the lower hinge of one heavy chain upon the binding of one FcγR molecule, which may preclude the binding of the second FcγR to the second heavy chain Fc thus providing an explanation to this 1:1 stoichiometry of the interaction.¹² Except for FcγRII, all known FcR are members of the Ig superfamily. The crystal structures of the extracellular domains of FcγRII,^{13,14} and FcγRIII¹⁵ show remarkable similarity. The receptors consist of two extracellular Ig-like domains, D1 and D2, with acute interdomain hinge angles of 50-55°, unique to Fc receptors, and with a Fc-binding region located in the D2 domain. The recent crystal structure of the FcγRIIb-Fcγ1 complex^{16,17} has revealed that the receptor-ligand interface consists of the BC, C'E, FG loops and the C b strand of the D2 domain, the hinge loop between the D1 and D2 domains of the receptor providing additional interactions with Fcγ (Figure 2). The receptor binds asymmetrically to the lower hinge region of both Fc heavy chains, creating a 1:1 receptor ligand stoichiometry.¹⁷ Low affinity FcγR have a low affinity for monomeric IgG but their biological role is to bind immune

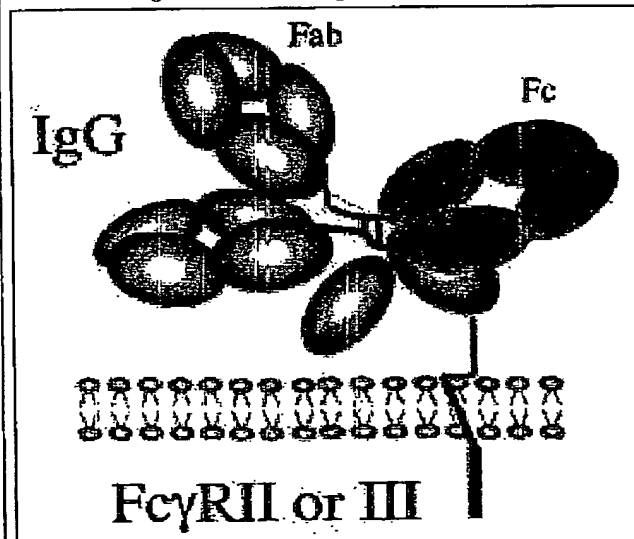


Figure 2
Schematic view of IgG-FcγRII or FcγIII interaction

complexes. Parallel dimers have been found in FcγRIIb crystal lattice. Their biological existence still has to be proven. However, it is possible that dimerization increases the avidity for immune complexes and subsequently facilitates cell activation.

Roles of Fcγ receptors in autoimmunity

Mice deleted for the ITAM-bearing signal-transducing γ chain associated with FcγRI and FcγRIII or for their respective ligand binding chains have impaired in vitro IgG-dependent phagocytic and ADCC responses (Table II).^{18,19,20} Since the γ chain is associated with the high affinity receptor for IgE, the γ-chain deficient mast cells are unable to respond not only to IgG but also to IgE. These mice are unable to mount type I and type

PHENOTYPE OF MICE DEFICIENT IN ACTIVATING RECEPTORS				
Deleted gene product		in vitro studies		in vivo studies
FcγR chain	FcγRI	ADCC	Phagocytosis	Functions impaired
	FcγRII	ADCC	Phagocytosis	Hypersensitivity reactions
	FcγRIII	ADCC	Phagocytosis	Resistance to autoimmune diseases
				Spontaneous (NZB/W mice)
				Induced by
				Collagen (DBA/1 mice)
				Antibodies
				Glomerulonephritis
				Vasculitis
				Alveolitis
				ITP
				AIHA

Table 2

PHENOTYPE OF MICE DEFICIENT IN INHIBITORY RECEPTORS				
Deleted gene product		in vitro studies		in vivo studies
FcγRIIb	FcγRIIb	ADCC	Phagocytosis	Functions Enhanced
	FcγRIIb	ADCC	Phagocytosis	Type I, II, III Hypersensitivity reactions
	FcγRIIb	ADCC	Phagocytosis	Antibody production
				Anaphylaxis (IgE, IgG)
				Susceptibility to autoimmune diseases
				Spontaneous
				(C57BL/6 mice) Glomerulonephritis
				Induced by
				Collagen (DBA/1 mice)
				MOS (EAE)
				Antibodies
				Glomerulonephritis
				Alveolitis

Table 3

Inhibitory receptors control inflammation and maintain peripheral tolerance (IC: Immune Complexes, EAE Experimental Autoimmune Encephalomyelitis)

III hypersensitivity reactions and are resistant to the induction of autoimmune diseases.²¹ In contrast, mice deficient for the FcγRIIb gene exhibit enhanced inflammatory responses in vitro and are prone to spontaneous and induced autoimmune



diseases (Table III).¹ These data obtained in mice have led to the concept that many systemic autoimmune diseases are under FcγR control (Figure 3). Complement indeed plays a crucial role in autoimmune-mediated inflammation. Most probably, activating FcγR control autoimmune reactions by increasing the uptake of immune complexes and by triggering effector macrophages whereas the inhibitory receptors control the activation of autoreactive B cells and thus maintain peripheral tolerance. In addition, FcγRIIb regulates the clearance of immune complexes by mononuclear phagocytes present in the spleen and liver. In the human, variants of low affinity FcγRIIIa and FcγRIIIa exist, with reduced affinity for immune complexes due to mutations in or near the IgG-binding site.^{22,23} Linkages between such FcγR polymorphisms and autoimmune diseases such as SLE, RA, Guillain Barré syndrome, and multiple sclerosis have been described. In addition, polymorphism in the FcγRIIIb-NA antigens (related to FcγR glycosylation) seem also to be involved in systemic autoimmune diseases.

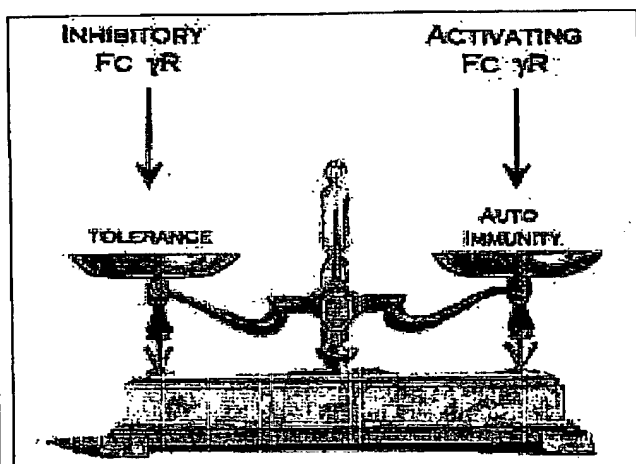


Figure 3
FcγR control the Tolerance-Autoimmunity balance

Roles of Fcγ receptors in antibody-based therapies

There has been a renewed interest in the last few years in the use of monoclonal antibodies (mAbs) in the diagnosis and treatment of various tumors²⁴ (Table IV). The most impressive clinical results have been obtained with Rituximab, a chimeric anti-CD20 mAb in the treatment of B cell lymphoma.²⁵ Herceptin, a humanized mAb that recognizes the human oncoprotein HER-2/neu overexpressed in some breast cancers and other tumors, induces clinical responses.²⁶ Other mAbs such as Campath-1H or 17-1A produced encouraging results in the treatment of chronic lymphocytic leukemia²⁷ or colorectal carcinoma²⁸ respectively. There is increasing evidence that the Fc portion of the anti-tumor mAbs is a major component of their therapeutic activity, through binding to FcγRs expressed by effector cells present in the tumor microenvironment. The polymorphisms of FcγRIIIb (Val/Phe¹⁵⁹) and FcγRIIIa (His/Arg¹³¹) that affect binding of IgG-immune complexes predict the response to Rituximab in patients with follicular lymphoma, supporting the hypothesis that ADCC by NK cells and

macrophages plays an important role in the clinical effect.^{29,30} As demonstrated in Fcγ receptor-deficient mice, the anti-tumor effects of Rituximab and Herceptin require the presence of the signal transducing γ chain to activate FcγRI and FcγRIII expressed on monocytes/macrophages and NK cells and are down regulated by inhibitory FcγRIIb at the monocyte/macrophage level.³¹ The improved efficacy in tumor eradication of bispecific molecules (BSMs) that have one arm specific for tumor cells and the other specific for FcγRs on immune effector cells, further illustrates the major role of FcγRs in mAb immunotherapy.³² The FcγR-dependent biological activities of therapeutic mAb's can indeed be extended to polyclonal IgG. As shown in experimental systems and in man, the efficacy of polyclonal IgG preparations from normal individuals (Intravenous Immunoglobulins) that are currently been used for therapy of many autoimmune diseases depends upon their interaction with host's FcγR.^{33,34}

In view of their pivotal role in the activation and regulation of IgG-dependent effector responses, FcγR provide new tools not only to predict the response to antibody-based therapies but also to manipulate the patient's response to treatment.

Molecular Target	Antibody	Indication	Nbr of clinical trials ^a
CD20	Rituximab	Chronic lymphocytic leukemia	71 (24%)
HER2/neu	Trastuzumab Pertuzumab	Metastatic breast cancer	42 (15%)
VEGF	Bevacizumab	Metastatic colorectal cancer	32 (11%)
CD52	Alemtuzumab (CAMPATH-1H)	Chronic lymphocytic leukemia	20 (7%)
CD25	Daclizumab (17-1A)	Chronic lymphocytic leukemia	12 (4%)

Table 4
Clinical trials based on MAb therapy (source Sept 2003
<http://clinicaltrials.gov>)
(* Percentage of the total MAb based trials, ITP: Idiopathic Thrombocytopenic Purpura)

Ectopic expression of FcγR on non-hematopoietic tumor cells

The first studies indicating that non-hematopoietic tumors may express FcγRs were performed on a variety of experimental tumors³⁵ and human cancers.^{36,37} However, the ectopic expression of FcγRs by non-hematopoietic tumor cells was controversial because the presence of inflammatory cells was quickly demonstrated at the tumor site³⁸ and because FcγR expression was lost during short-term culture of tumor cells *in vitro*.³⁹ In the last few years, studies were initiated analyzing the expression of FcγRs by tumor cells of non-hematopoietic origin. The studies demonstrated that tumor cells from about 40 percent of human metastatic melanomas tested expressed inhibitory FcγRIIb1 *in vivo* and *ex vivo*.⁴⁰ In earlier studies using Polyoma virus-induced mouse tumors expressing FcγRIIb1, it was shown that this receptor confers an *in vivo* growth advantage to tumor cells and increases their malignancy.⁴¹ It was hypothesized that increased tumorigenicity mediated by FcγRIIb1 could involve immunological mechanisms. For



example, FcγRIIb1 expressed by tumor cells could block complement-dependent lysis of tumor cells or could protect tumor cells from ADCC by binding the Fc portion of Abs covering tumor cells.⁴ Recently, it has been shown that FcγRIIb1 expression by human metastatic melanomas has a profound down regulatory impact on tumor growth and uptake in nude mice. This effect is under the control of IgG anti-tumor antibodies.^{4b}

Conclusion

The knowledge about FcγR functions and structure has indeed progressed a lot since their original discovery in the 70s.^{4,4A,4B} Not only are FcγR important molecules that mediate and control the effector functions of IgG antibodies, but they also control the autoimmunity-tolerance balance in the periphery. Furthermore, they are major agents of the efficacy of therapeutic antibodies. The recent description of the crystal structure of the FcγR/Fc complex opens new possibilities to manipulate FcγR/Fc interactions and hence the efficacy of such antibodies.

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